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**THE ACTIVATION OF PEPSINOGEN**

by

**Larry Chi-Yang Yuan, M.Sc.**

**A Thesis**

**Submitted in Partial Fulfillment of**

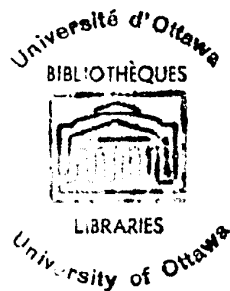
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**Degree of**

**Doctor of Philosophy**

**University of Ottawa**

**March, 1963**



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## PREFACE

Precursors of biologically active proteins are known as zymogens or proenzymes. Zymogens are inactive precursors of the corresponding enzymes, and may be activated in various ways. Elucidation of the mechanism of activation, and of the differences between the precursor and the corresponding active enzyme, may yield results of far-reaching importance on the nature and the action of the enzymes.

The activation of trypsinogen and chymotrypsinogen has recently been studied in considerable detail, but the nature of the process of the activation of pepsinogen has not yet been completely cleared up; the observed changes are somewhat complicated, and it is not clear how far they are associated with the activation itself.

The activation of pepsinogen appears to involve a fragmentation of peptides; if those peptides can be isolated and determined, it will give an important clue to the difference between the two proteins and also to the nature of the structure of the enzyme.

The fluorodinitrobenzene method of Sanger has been used extensively during the past decade, and is now a well-established procedure in protein chemistry. It can be applied to study the structure of small peptides isolated from partial hydrolysates of proteins and polypeptides. The condensation with the reagent is quantitative and relatively free from side reactions, and the products are bright yellow which is very helpful for their chromatographic separation.

Therefore, we have attempted to analyze the activation mixtures by the FDNB method, and to study the chemical events occurring during the conversion of pepsinogen to pepsin.

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CODE OF ABBREVIATIONS

FDNB	1-Fluoro-2, 4-dinitrobenzene
DNP-derivatives	Dinitrophenyl-derivatives
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Cys	Cystine
Gly	Glycine
Glu	Glutamic acid
His	Histidine
Ileu	Isoleucine
Leu	Leucine
Lys	Lysine
Meth	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Tryp	Tryptophane
Tyr	Tyrosine
Val	Valine
W <sub>1</sub> , W <sub>2</sub> , W <sub>3</sub> , W <sub>4</sub> and W <sub>5</sub>	DNP-peptides formed in water layer
E <sub>1</sub> , E <sub>2</sub> , E <sub>3</sub> and E <sub>5</sub>	DNP-peptides formed in ethyl acetate layer

ABSTRACT

The activation of purified pepsinogen was studied at pH 2.0 and 5.6 and at 20°C and 0°C. It was found to be catalyzed by hydrogen ions at pH 2.0 and to be truly autocatalytic only at pH 5.6 and 0°C. A certain number of peptides produced during activation were isolated, the rate of formation of the main peptide was found to be identical with the rate of formation of pepsin. The production of the other important peptides could be explained only by assuming that more than one species of active pepsin is produced during activation. It is concluded that the production of each species of active pepsin involves the cleavage of only one peptide bond in each case.

## CHAPTER 1. INTRODUCTION

### (1) General considerations of the mechanism of activation of proteolytic zymogens

The process of activation of zymogens has certain common features. The transformations are catalyzed by proteolytic enzymes which operate with a high degree of specificity and selectivity, effecting the hydrolysis of a limited number of peptide bonds in the zymogen molecule. The product of activation may be considered as an enzymatic modification of the precursor.

The hydrolytic cleavage of one or more peptide bonds in the zymogen molecule is an essential feature of these reactions, therefore, many chemical changes may be expected. Each peptide bond which has been hydrolyzed will give rise to one N- and C-terminal group which may be determined by one of the methods of end group analysis. If the hydrolyzed bond is in a terminal region of a polypeptide chain of the zymogen molecule, an amino acid or peptide fragment will be formed, and can be determined by separating the protein and peptide or amino acid fractions from one another. If the hydrolyzed peptide bond occupies an internal position in the polypeptide chain, cleavage of the protein molecule into relative large fragments may occur.

A detailed comparison of the chemical and physical properties of the zymogens before and after activation is essential for elucidating the nature of the changes occurring during activation. The modern

methods of protein chemistry and enzymology are available for structure studies, and a number of contributions are using a variety of means to uncover the structure changes during the activation of zymogens. The dramatic development of recent years in the study of activation of trypsinogen and chymotrypsinogen provided the most striking set of examples of activation of proteolytic zymogens.

a. The activation of trypsinogen

The N-terminal sequence of trypsinogen is Val. (Asp)<sub>4</sub>. Lys. Ileu. Val. Gly (1, 2, 3). It has no detectable C-terminal residue (4, 5). Thus it is likely to contain one open chain with a covalently blocked  $\alpha$ -carboxyl group. A series of sequences have already been elucidated in this chain (6).

Trypsin is formed under a variety of conditions. The activation is caused by three different enzymes, i.e. trypsin itself, enterokinase, and mold proteinases.

In the pH range of 5.0 - 9.0, the addition of trypsin catalyzes the transformation of trypsinogen into two products, trypsin and an inert protein (7). Both of these reactions give S-shaped progress curves; in the case of the formation of trypsin this is due to the autocatalytic nature of the reaction.

It was noticed that some acid solutions of trypsinogen became activated quite rapidly. This abnormal activation in certain acid solutions was traced to the presence of a minute amount of mold, penicillium. It was found on cultivating the mold that the culture medium

became increasingly powerful in its ability to form trypsin from trypsinogen, and a concentrated and partially purified solution of this activating principle has been obtained. It is associated with a proteolytic enzyme produced by the mold, but whether or not it is identical with this enzyme has not yet been determined. In any case, the substance presents a very useful reagent for the formation of trypsin from trypsinogen, since the reaction occurs in acid solution under which condition both trypsin and trypsinogen are stable. The optimum pH is about 3.0.

The activation of trypsinogen by enterokinase (7, 8, 9) is usually accompanied by two other competing reactions, namely, the autocatalytic formation of trypsin and the formation of inert proteins. However, in solutions with more acid than pH 6, the rate of formation of inert proteins is minimized, whereas the autocatalytic reaction can be made negligible in dilute trypsinogen solutions.

Since the molecular weight of trypsinogen and trypsin, as determined by sedimentation and diffusion (10, 11), or as calculated from the amino acid analysis (12), is practically the same; it is clear that no large protein fragment has been split off during the autocatalytic activation of the trypsinogen. The first indication for the liberation of a peptide was provided by the findings of Desmuelle and coworkers (13) that each of the two proteins has only one N-terminal

group, which, however, is different in the two cases, suggesting that a peptide fragment had been liberated from the N-terminal region of the molecule. The higher isoelectric point of trypsin, as compared to trypsinogen, suggested that such a peptide must have predominantly acidic properties. The amount of peptide liberated was reported to be proportional to the degree of activation (14), thus indicating the liberation of this peptide and the appearance of tryptic activity are related phenomena. Quantitative amino acid analysis of acid hydrolyzates of the purified peptide yielded aspartic acid, valine, and lysine in mole ratios of 4:1:1. The structure Valyl-(Aspartyl)<sub>4</sub>-Lysine was assigned to this hexapeptide.

All evidence at present available indicates that one, and only one, peptide bond is split during the activation of trypsinogen, resulting in the liberation of a hexapeptide. Since this hexapeptide is derived from the open end of the single peptide chain of the molecule, it is difficult to visualize how this event in itself can be the cause for those structural changes in the molecule which are responsible for the appearance of enzymatic activity. However, it may be significant in this connection that the N-terminal portion of trypsinogen which is removed during activation in the form of a hexapeptide contains four adjacent negative charges contributed by the  $\beta$ -carboxyl group of the adjacent aspartic acid side chains. Electrostatic repulsion might maintain that segment of the peptide chain in an extended configuration (15) and exclude it from orientation in a helical pattern. This fact,

in turn, may be responsible for the susceptibility of the lysyl-isoleucyl bond to tryptic hydrolysis during activation in preference to all other bonds which might be protected by being hydrogen-bonded. As a further consequence of the particular composition of the N-terminal sequence of trypsinogen, it may be assumed that one or more of the aspartic acid side chains is hydrogen bonded to more distant side chains along the polypeptide chain of trypsinogen, thus establishing a loop. Splitting of the lysyl-isoleucine bond would remove the structure rigidity in this region and allow one of the newly formed chain ends to assume a more nearly helical configuration. According to this interpretation, the structural contribution of two or more hydrogen bonds, or salt linkages, is an important factor in the conversion of the trypsinogen to the active trypsin.

b. The activation of chymotrypsinogen

The cattle pancreatic juice contains two chymotrypsinogens, chymotrypsinogen A and chymotrypsinogen B. There is very close agreement between physicochemical properties, the most striking difference between the proteins is only the isoelectric point which is 9.1 for chymotrypsinogen A and 5.2 for chymotrypsinogen B at 0.1 ionic strength. Chymotrypsinogen A can be easily crystallized and recrystallized in high yield from ammonium sulfate solution or aqueous ethanol. Chymotrypsinogen B does not crystallize so readily (16), and it was originally thought to be of minor importance.

The molecular weight of chymotrypsinogen A is 25,000; the molecular weight of chymotrypsinogen B is likely to be about the same.

When the techniques for end group determination are applied to chymotrypsinogen A in their ordinary form, no terminal residues are found. Thus the protein was considered as cyclic for some years. In fact, it represents a curious combination of two special cases. The N-terminal residue is half cystine, which can not be found before performic acid oxidation (17). The C-terminal residue is asparagine (18, 19, 20), which can not be detected by hydrazinolysis and which is unavailable to carboxypeptidase in the native protein. The C-terminal sequence becomes available after destruction of the S-S bridges. The reduction does not modify the protein molecular weight (6). This result suggests that chymotrypsinogen A contains a single open chain beginning with half cystine and ending with asparagine.

The activation of chymotrypsinogen by trypsin can give rise to several enzymatically active components, the pathway and the final product depending on the concentration of trypsin. Two conditions of activation may be distinguished, usually referred to as fast and slow activation. Under conditions of fast activation (21), maximal proteolytic activity is attained at 0°C after 1 - 2 hour; whereas under conditions of slow activation (22), the maximal activity is reached at 0°C after 24 hours. Slow activation is the process originally described by Kunitz and Northrop (22) for the conversion of chymotrypsinogen into crystalline  $\alpha$ -chymotrypsin and other proteins which after further

activation yielded the crystalline products,  $\beta$  and  $\gamma$ -chymotrypsin (23). The products of rapid activation have been denoted as  $\pi$  and  $\delta$ -chymotrypsin. The end products of these two activation processes are presumably not interconvertible. The rates of activation processes are controlled by the trypsin concentration. The chymotrypsinogen-trypsin weight ratio is 36:1 in the case of the rapid activation, and approximately 10000:1 in the case of the slow activation.

It seems reasonably certain that during rapid activation of chymotrypsinogen two peptide bonds are hydrolyzed. At first, chymotrypsinogen-A is converted by trypsin into  $\pi$ -chymotrypsin by the splitting of an arginyl-isoleucine bond. The splitting of a single bond is associated with the appearance of enzymic activity;  $\pi$ -chymotrypsin is actually the most active of all known chymotrypsins. Then  $\pi$ -chymotrypsin autolyzes (24); the second bond is broken off from one of its C-terminal sequences, giving rise to  $\delta$ -chymotrypsin and to a dipeptide, serylarginine. This minor change does not influence the activity. And then the process comes to a stop,  $\delta$ -chymotrypsin being the relatively stable end product of rapid activation.

According to the investigations of Kunitz and Northrop (22), the slow activation of chymotrypsinogen by trypsin occurs at maximum rate between the limits of pH 7 and 9, the rate being decreasing rapidly on either side of this range. Two additional processes (25) have time to take place. Chymotrypsinogen is slowly attacked by chymotrypsin, and  $\delta$ -chymotrypsin slowly autolysis. Two bonds, tyrosyl-threonine and

asparaginy-l-alanine, located in another region of the molecule are split, and a second dipeptide, threonylasparagine, is liberated. When these chymotrypsin-catalyzed splittings take place alone, that is to say in the absence of trypsin, no activation occurs. Chymotrypsinogen A is converted into new and still-activable forms which have been called neochymotrypsinogens(25). When trypsin is present in small amount, as is the case during the slow activation, two possibilities exist: the trypsin-catalyzed activation of chymotrypsinogen, followed by  $\delta$ -chymotrypsin spontaneous autolysis, and chymotrypsin-catalyzed conversion of chymotrypsinogen into neochymotrypsinogens, followed by a trypsin-catalyzed activation of these latter substances. Both processes involve the splitting of a total four bonds and give rise to  $\alpha$ -chymotrypsin and two dipeptides, serylarginine and threonylasparagine. The  $\alpha$ -chymotrypsin containing N-terminal isoleucine also contains alanine as an N-terminal group (13), and tyrosine and leucine as C-terminal groups (26, 27).

## (2) Chemistry of pepsinogen and pepsin

### a. Methods of preparation

The isolation of pepsinogen from gastric mucosa consists in the extraction of the tissue with 0.45 saturated ammonium sulfate, containing sodium bicarbonate, and precipitation of protein at 0.63 saturated ammonium sulfate. The precipitate is then absorbed on cupric hydroxide at pH6 and pepsinogen eluted with 0.1 M phosphate buffer, pH 6.8. After repeating this step, pepsinogen crystallizes from 0.4 saturated ammonium sulfate at pH 6.25 (3, 23).

Pepsin is produced from pepsinogen autocatalytically. Acidification of pepsinogen solution is sufficient to bring activation, since traces of pepsin are always present.

Pepsin may be purified from gastric juice (8), from crude commercial preparations (10), or from pepsinogen obtained from gastric mucosa (29). The first crystalline pepsin was obtained from swine by a salt fractionation procedure (30) which has since been somewhat modified (29, 31).

A preparation low in salt is obtained by crystallization from 20% alcohol (32). Commercial crystalline pepsins often contain significant non-protein contaminants, probably peptides arising from autolysis (33). Heirwegh and Edman (34) removed peptide contaminants from pepsin preparations by passing a 0.5% solution of pepsin at pH 2.9 through a Dowex-50 column at low temperature. Mits and Schlenker (35) have employed absorption on the weak anion exchanger DEAE-cellulose (36) to obtain a stable dry product.

Oreckovich et al. have purified pepsin with chromatography on DEAE-cellulose (37, 38). Arnon and Perlmann (39) have reported that the procedure for the purification of pepsinogen can be appreciably simplified with the use of chromatography on DEAE-cellulose column, and commercial crude pepsinogen has been purified by a modification of the procedure of Ryle (40).

#### b. Heterogeneity of pepsin

Although swine pepsin was the first of the proteinases to be

crystallized, it has been clear for years that the usual crystalline preparations do not represent a homogeneous material.

Crystallized pepsin has been successfully split by adsorption on alumina into fractions, one of which is characterised, particularly by a liquefying action, and the other by a more hydrolyzing action on gelatin (41); Northrop (42) has also indicated the presence of an enzyme constituent in crude pepsin preparations which liquefies gelatin.

Herriott et al. (43) have reported the preparation of several active protein components from crystalline swine pepsin. These authors have obtained a pure pepsin-A of constant solubility from pepsinogen and from certain commercial pepsin preparations. It has proved impossible to separate the pepsin-A from the other protein components by means of electrophoresis at pH 4.1 to 5.2.

Neumann and Sharon (44) described the preparation of a pepsin, denoted as pH-3 pepsin which does not exhibit transpeptidation activity and yet preserves its hydrolytic activity.

pH-3 pepsin and pH-2 pepsin have similar activities toward hemoglobin. They differ markedly, however, in their behaviour towards synthetic substrates.

The pH-3 pepsin could readily be converted to pH-2 pepsin by lowering the pH to 2 with HCl.

A fragment of pepsin of molecular weight below 10,000 with one third of the original activity, has also been isolated by Tokuyisu and Funatsu by chromatography on Amberlite IRC-50 and Dowex 50X2 (45).

Crystalline swine pepsin prepared from commercial preparations or from pepsinogen (28) is not a single protein as judged by solubility measurements. The solubility behavior of the protein resembles that of a solid solution, but at pH 5 a more soluble fraction could be isolated showing constant solubility in two or more different solvents (8).

Using various protein substrates, Northrop (46) observed a broad pH region of peptic activity extending from below pH 2 to above pH 4 with a maximum at pH 1.6. In contrast, when synthetic substrates were used, maximum hydrolytic activity occurred at pH 4 (47, 28). This discrepancy in pH optima might be attributed to the presence of more than one pepsin in crystalline pepsins.

#### c. Specificity of pepsin

The action of pepsin on synthetic substrates has not been measured with the same precision as has the hydrolysis of synthetic substrates by other proteolytic enzymes. Pepsin hydrolyses only peptide linkages; it is not an esterase and will not attack amide linkage. Amino acid must be of the L-configuration on both sides of the peptide bond, and aromatic residues greatly favour attack.

In contrast to its slow action on several synthetic peptides, pepsin acts rapidly on a wide variety of peptide bonds in protein substrates. The evidence on this point is largely the result of the work of Sanger and coworkers (49, 50) on the peptic digestion of the A and B chains of oxidized insulin. The results are summarized as follows:

<u>Slowly attacked bonds</u>	<u>Rapidly attacked bonds</u>	<u>Related bonds not attacked</u>
Phe-Val	Phe-phe	Leu-val
Tyr-glu	Phe-tyr	Isoleu-val
Val-cys SO <sub>3</sub> H	Leu-tyr	Val-asp
Ala-leu	Leu-glu	Val-glu
Glu-his	Glu-asp	Gly-glu
Glu-leu		Asp-glu
Gly-phe		Asp-tyr
Glu-ala		
Glu-glu		

It is true that the bonds between two phenylalanyl residues and phenylalanine and tyrosine were split rapidly, but so also was the bond joining a leucine to valine. The high lability of the leucyl-valyl bond may have been unusual, since another leucyl-valyl bond was not split. From this evidence it would appear that either the surrounding amino acids have an influence or there is some special strain on such leucyl-valyl bond which makes it labile.

In other proteins or polypeptides of known amino acid sequence, similar unforeseen results have been reported.

In ribonuclease (51), the two -ala-ala- bonds in -ala-ala-ala- sequence were split by pepsin along with -phe-glutamine and -phe-glutamic acid.

In  $\alpha$ -corticotrophin (52), it was the -ala-ser- bond. This shows that the specificity of pepsin is very broad and local conditions

of stress on these bonds may be critical.

d. Physicochemical properties

The molecular weight of pepsinogen (28, 53) derived from osmotic pressure measurement is  $42,000 \pm 3,000$ . Electrochemical characterization is limited to the determination of the isoelectric point by the microscopic cataphoresis method, pH 3.6 - 4.3 (28). It is considerably less acidic than pepsin, and is stable in dilute alkali, pH 7 to 9. It is destroyed on 24-hour standing in 50% aqueous ethanol (54) and is stable for only short periods below pH 6 (28).

Neutral, salt-free solutions of pepsinogen, may be heated to boiling and then cooled without loss of potential activity. If salt is added to the hot solution, however, it is found that the protein is entirely denatured in the hot solution, but in the absence of salts reverts to the native condition again when cooled (8).

Pepsinogen is denatured by strong alkali, i.e. pH greater than 9.0, but this reaction is also reversible, at least to a certain extent (8).

The molecular weight of pepsin by the measurements of light scattering (55), film pressure (56), osmotic pressure (30), and x-ray diffraction (57), seems to point toward 34,500 as the most probable value.

The pure pepsin is believed to have an isoelectric point below pH 1 (58), a value which is in accord with the low content in basic amino acid residues.

Pepsin gradually loses activity in strongly acid solution (59, 60). At pH 1.8 and 50°C, loss of activity is paralleled by loss of protein nitrogen; under these conditions, autolysis probably also contributes to the loss of activity (60). In very strong acid, there appears to be first a denaturation, followed by hydrolysis of peptide bonds (60).

Pepsin is inactivated at alkaline pH values, and the loss of activity is not readily reversible (61).

e. Chemical composition

(a) Amino acid composition

The amino acid composition of pepsinogen and pepsin have been determined recently (39, 62, 63).

Pepsinogen: Asp (46), Glu (30), Gly (36), Ala (21), Val (25), Ileu (25), Leu (33), Ser (47), Thr (28), Half-Cystine (6), Meth (4), Pro (19), Phe (15), Tyr (17), Tryp (6), His (3), Lys (11), Arg (4). Total amino acid residues per mole is 376.

Pepsin: Asp (44), Glu (27), Gly (38), Ala (18), Val (21), Ileu (27), Leu (28), Ser (44), Thr (28), Half-Cystine (6), Meth (5), Pro (15), Phe (14), Tyr (18), Tryp (6), His (1), Lys (1), Arg (2). Total amino acid residues per mole is 343.

It is worth noting here that on conversion to pepsin there is a disproportionate loss of basic amino acids. This, coupled with the finding that in pepsinogen the  $\epsilon$ -amino groups of the twelve lysines are free (62), may have special significance for the greater stability of pepsinogen than pepsin in weakly alkaline solutions.

(b) N- and C-terminal sequences

End group analyses show that both pepsin and pepsinogen consist of only one chain. The N-terminal residues of pepsinogen and pepsin, determined by reaction with fluorodinitrobenzene (64), are found to be leucine and isoleucine. That, the N-terminal residue of pepsin is isoleucine, has been confirmed by Heirwegh and Edman (34). The N-terminal sequences, determined by digesting the dinitrophenyl (DNP) derivatives for short periods with 6N HCl and identifying the DNP amino acid and DNP peptides (64), are different for these two proteins, and so pepsin can not occupy the N-terminal position. Williamson and Passmann (65) determined the N-terminal hexapeptide sequence of pepsin, using the Edman phenylisothiocyanate degradation (66). They reported the N-terminal residue to be leucine instead of isoleucine.

The C-terminal group in pepsinogen, as determined by the use of carboxypeptidase, was found to be alanine (67). The adjacent C-terminal amino acids are leucine and valine (67). The C-terminal sequence of pepsin has been determined by Van Vunakis and Herriott (63) and by William and Passman (69), using carboxypeptidase. For both molecules, the sequence is -val.leu.ala; pepsin, therefore, constitutes the C-terminal part of the pepsinogen molecule.

(c) Disulfide bonds and phosphate binding

Kern (67, 70), using monothioglycol as reducing agent, found that two disulfide bonds can be readily reduced in both pepsin and pepsinogen, and that there is a third which is very difficult to reduce.

After reduction of all three cystine disulfide bridges, the protein yielded the same osmotic pressure as the native untreated protein. This evidence supports the single peptide chain concept for the structure of pepsinogen.

Perlmann (58) has reported that the phosphorus is bound as a diester linking two portions of the chain in pepsinogen or pepsin molecule, and is nonessential for the biological activity of these proteins.

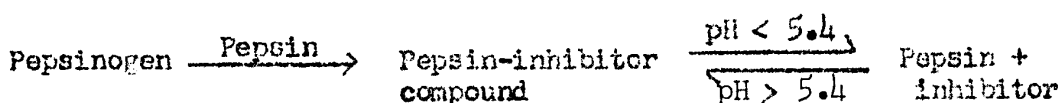
(d) Conversion of pepsinogen to pepsin

Conversion of pepsinogen takes place in solutions which are more acidic than pH 5. This reaction is autocatalytic; i.e., one of the products (pepsin) catalyzes the conversion. Proof of this conclusion was obtained by observing an increased rate of activation upon addition of pepsin to the mixture (28).

Seven to nine (62) peptide bonds are hydrolyzed during the formation of pepsin, but if the single chain concept is correct, it is probable that cleavage of only one of these bonds is required to release the active enzyme, and that the other six to eight bonds may reflect other susceptible linkages, unrelated to the release of activity. It would appear likely that at least one peptide bond must be split during conversion, for pepsinogen is not active and pepsin which catalyzes the conversion cleaves only peptide bonds.

The activation process is, however, complicated by the circumstance that the compound (or one of the compounds) split off from

pepsinogen acts as an inhibitor for the pepsin formed (71). Herriott gave the following scheme:



The inhibitor is unstable to alkalis. It is split by pepsin, soluble in boiling 2.5% trichloroacetic acid, and dialyses slowly through celloidion membranes.

That the activation of pepsinogen is an ordinary proteolytic reaction with no intrinsic specificity is known by the fact (72) that swine pepsin transforms chicken pepsinogen into chicken pepsin and vice versa.

Herriott (73) has described the crystallization of a polypeptide which inhibits the milk-clotting activity of pepsin at pH 5.3. The inhibitor is derived from pepsinogen upon its autocatalytic conversion into pepsin. The reversible combination of pepsin with the inhibitor appears to follow quantitatively the simple mass law. The inhibitor does not retard pepsin at pH 2.0, and appears to be digested by pepsin with a maximum rate near pH 3.5.

Van Vunakis and Herriott (74) isolated the inhibitor from activation mixtures with ion exchange resins and determined its amino acid composition. The FDNB method of Sanger was used for end-group determination, and the finding of only one N-terminal amino acid residue, identified as leucine, suggests that the molecule consists of a single peptide chain. The recovery of DNP-leucine would indicate a

value of 3,100 for the molecular weight of the inhibitor, while the value 3,242 was deduced from amino acid analysis, in agreement with previous determination.

The immunochemical properties of different pepsin and pepsinogen preparations have been studied by Lobachevskaya (75) who concludes that only limited structural changes occur during activation.

Lokshina and Orekhovich (76) studied the activation of DNP-pepsinogen. In order to obtain DNP-derivative, pepsinogen was incubated in the presence of FDND in phosphate buffer at pH 7.0 for 20 hours at room temperature. The preparation was dialyzed for two days and then frozen. Activation proceeded at room temperature for 3 minutes and was cut short by the addition of 1 N NaOH until an alkaline reaction was obtained. The activation of DNP-pepsinogen and presumably pepsinogen is accompanied by the splitting off a peptide from the N-terminal portion of the molecule. In addition, the appearance of other peptides was observed upon activation.

Lokshina and Orekhovich (77) have established the amino acid sequence of the N-terminal portion of pepsinogen, involving eighteen residues: Leu-val-leu-glu-pro-ala-glu-phe-ser-leu-lys-asp-gly-lys-val-(asp, pro)-leu .

Recently, Herriott (78) has presented an activation scheme, according to which the removal of one peptide from pepsinogen would set free the active site of pepsin. A tyrosine phenol group and a free carboxyl group were tentatively assigned to the active group.

CHAPTER 2. EXPERIMENTAL PROCEDURES

(1) Purification of pepsinogen

Sephadex G-25 was used in this work. It is the trade name of a cross-linked polysaccharide dextran made by Pharmacia Uppsala, Sweden. The manufacturer's specification for the Sephadex G-25 was as follows: Lot No. To 6359; dry screen analysis on U.S. No. 50: 0.0 percent; through U.S. No. 270: 5 percent; water regain: 2.7 gm. water per gm. dry gel.

10 gms. of Sephadex G-25 was allowed to swell in phosphate buffer (pH 6.8, 0.001 M) for half an hour and was then freed from fine grained material by repeated sedimentations and decantations. The gel grains were poured into a column (1.8 by 30 cm). A circular filter paper was put on the top of the column to protect the surface.

50 mg. crystalline pig pepsinogen (Nutritional Biochemicals Corporation, Cleveland, Ohio) were dissolved in 4 ml. phosphate buffer (pH 6.8, 0.001 M) and a small amount of insoluble material centrifuged off. Then the pepsinogen solution was applied on top of the column; and when it had completely entered the bed, the column was washed with phosphate buffer at a constant rate of 2 ml. per minute. The effluent was collected in 1 ml. portions. The appearance of protein and peptide impurities in the fractions was followed by paper chromatography. Pepsinogen appeared as a single component remaining at the origin on the paper. Fractions 15 to 27 contained pure pepsinogen. The remaining

material was removed by washing with excess buffer, then the column was suitable for another run. The effluents were quickly frozen and lyophilized; only 44 percent of the original material was recovered as pure pepsinogen.

(2) Activation

200 mg. of the freeze dried pure pepsinogen was dissolved in 30 ml. of distilled water. The solution was adjusted rapidly to pH 5.6 or pH 2.0 at 20°C or at 0°C. After a certain period of time the pH of the solution was raised to 8 by addition of NaHCO<sub>3</sub>.

(3) Determination of the remaining pepsinogen in the activation mixture

5 ml. samples were taken out of activation mixture and immediately brought to pH 8 by addition of NaHCO<sub>3</sub> solution. After standing at room temperature for 30 minutes, the samples were activated again with HCl at pH 2 and 20°C for 20 minutes. The activity of the newly formed pepsin was then determined with the aid of the hemoglobin method at pH 2 (8).

(4) FDNB reaction

After the addition of NaHCO<sub>3</sub>, the activation mixture was shaken with 200 mg. of fluorodinitrobenzene (FDNB) (dissolved in 5 ml. ethanol) for 2 hours at room temperature. It was then extracted with several 15 ml. portions of ethyl ether to remove the ethanol and the excess of FDNB. The aqueous solution was acidified with a few drops of concentrated HCl. The precipitate was centrifuged down, and the

supernatant was extracted with three 15 ml. portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness in vacuo. The aqueous layer was frozen and lyophilized.

(5) Paper chromatography separation

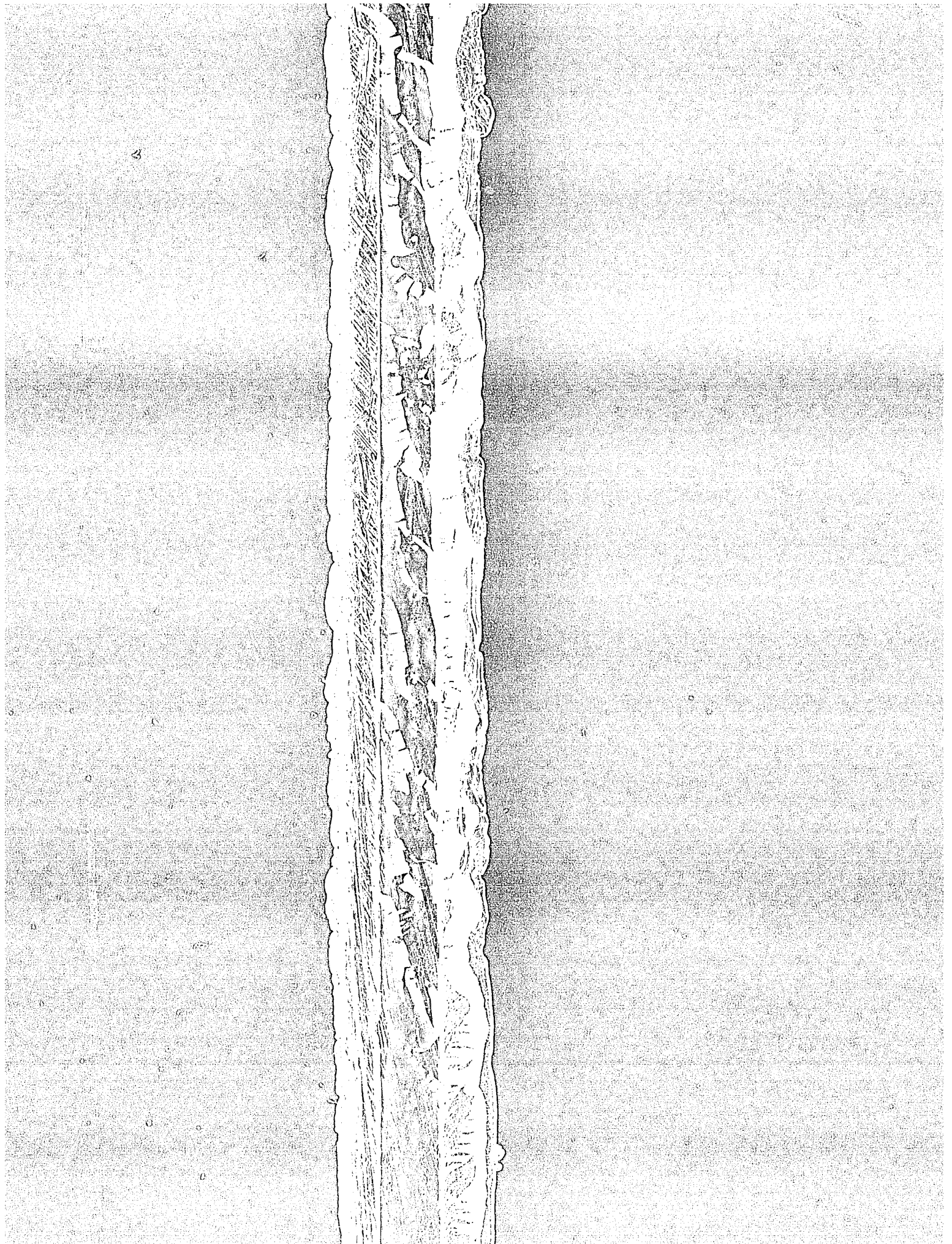
The residues from the ethyl acetate layer and the water layer were quantitatively transferred on the paper chromatogram. Whatmann No. 3 MM paper was used. The paper sheet was first sprayed with pH 6 phthalate buffer and allowed to dry. The chromatogram was then allowed to run for 48 hours (24 hours in the case of ethyl acetate residues) in the dark in the tert-amyl alcohol-phthalate system of Blackburn and Lowther (79).

(6) Extraction of DNP-derivatives from the paper

Yellow bands, present on the paper, were cut out, and the yellow substances eluted from the paper in the dark with 10 ml. of 1% NaHCO<sub>3</sub> solution. A blank paper strip of similar size was also cut and eluted with NaHCO<sub>3</sub> solution. The optical densities were determined in Beckman DU spectrophotometer at 350 mμ.

(7) Determination of the N-terminal amino acid of the DNP-derivatives

An equal volume of concentrated HCl was added to the DNP-derivative solutions, and the mixtures were hydrolyzed in sealed tubes at 105°C for 16 hours. The hydrolyzates were then diluted and the N-terminal amino acids were extracted with ethyl ether. The DNP-amino acids present in the extracts were identified by paper chromatography (79).



Regeneration of the free amino acids from the DNP-derivatives was achieved by hydrolysing the DNP-derivatives with barium hydroxide (80). Leucine and isoleucine were resolved in the tert-amyl alcohol-phthalate system described above.

(8) Semi-quantitative determination of the amino acid composition of the DNP-derivatives

After ether extraction, the aqueous layer of the hydrolyzate of the DNP-derivatives was evaporated to dryness. The residue was dissolved in water and treated with Dowex-50, and then the amino acids were eluted from the resin with 2 N  $\text{NH}_4\text{OH}$ . The eluate was evaporated to dryness, and spotted on Whatmann No. 1 paper. The paper chromatogram was first run for 30 hours in N-butanol-acetic acid-water (4:1:5). After drying, the paper chromatogram was run in the second direction in 90% phenol for 24 hours.

The dry paper chromatogram was sprayed with a 2% alcoholic ninhydrin solution. After 30 hours in the dark, all the spots on the paper were cut out. A piece of paper of the same size was also cut out to be used as a blank. The colour was eluted from the paper squares with ethyl alcohol and the clear solution read against the blank at 570  $\text{m}\mu$  (385  $\text{m}\mu$  for proline) in the Beckman DU Spectrophotometer (79).

(9) Determination of the C-terminal amino acids of the DNP-derivatives

a. Preparation of the carboxypeptidase

The crystalline carboxypeptidase suspended in water (50 mg. in 1 ml.) containing  $\text{NaHCO}_3$  was obtained from Worthington

Biochemical Corporation. The stock solution was prepared by diluting 0.1 ml. of suspension with 50 ml. of  $\text{NaHCO}_3$  solution (pH 8.0).

b. Reaction

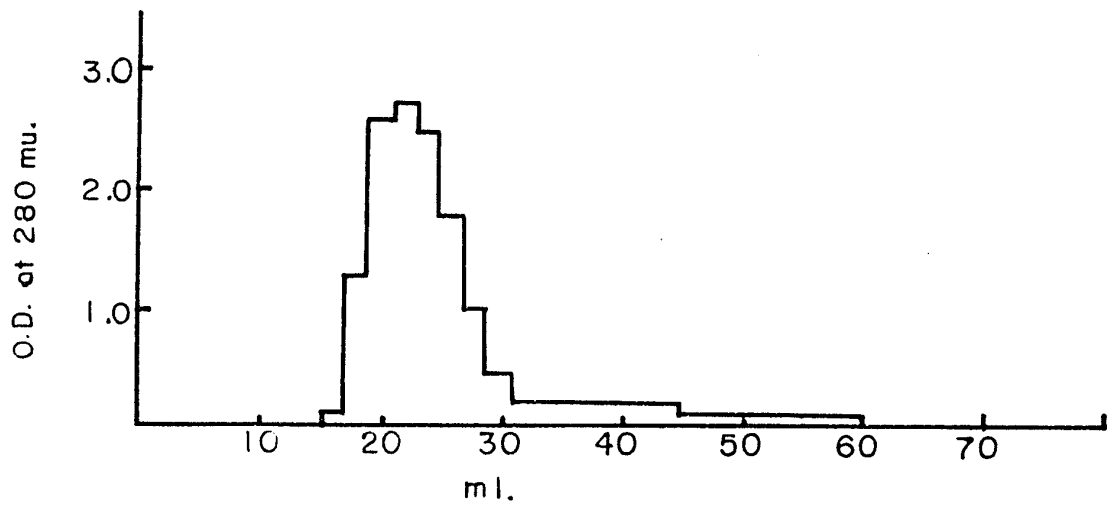
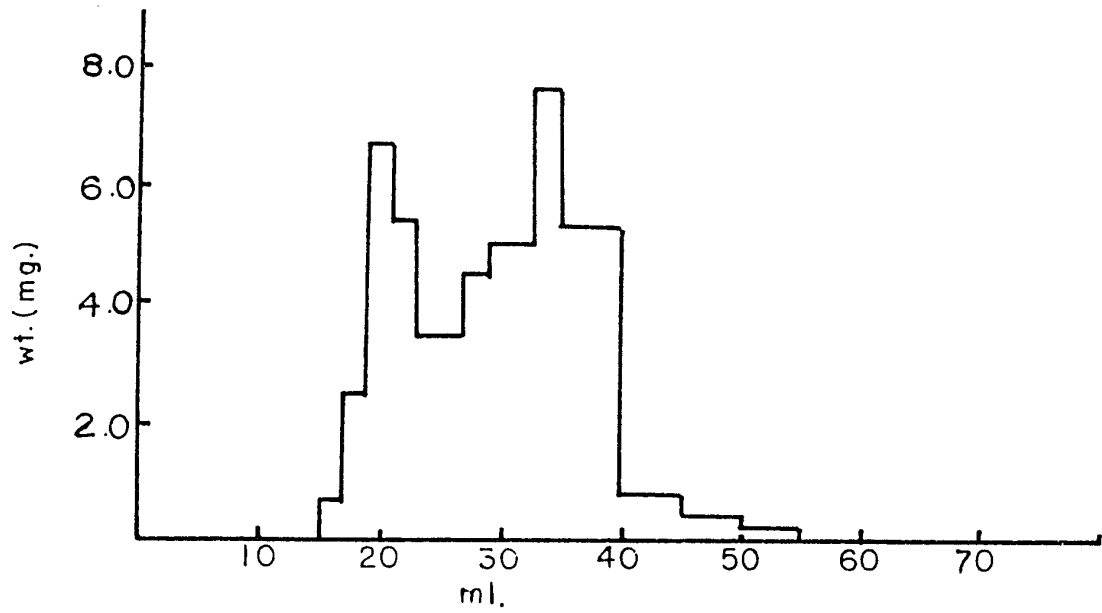
The DNP-derivatives (less than 2 mg.) were dissolved in 3 ml. distilled water and adjusted to pH 8.0. 0.1 ml. (substrate to enzyme ratio 50:1) of carboxypeptidase stock solution was added. Aliquots (0.5 ml.) were removed from the mixture after 15, 30, 45, 60, 90 minutes. A few drops of concentrated acetic acid were added to the samples to terminate the enzyme action. The free amino acids in the solutions were taken up on Dowax-50, and then eluted with 2 N  $\text{NH}_4\text{OH}$ . The eluate was evaporated to dryness and the amino acids present in the residue were analyzed qualitatively and quantitatively as described in section 8.

### CHAPTER 3. RESULTS AND DISCUSSION

#### (1) Purification of pepsinogen

The pepsinogen used in this work was a commercial preparation obtained from Nutritional Biochemicals Corporation. When the purity of this preparation was examined, it was found that the pepsinogen was very impure. The purity was tested by running paper chromatograms of pepsinogen solutions, or of pepsinogen-fluorodinitrobenzene reaction mixtures. In both cases the presence of 3 to 10 contaminants was detected on the paper. As the presence of these amino acid or peptide contaminants would have certainly obscured the identification of the chemical changes occurring during the activation of pepsinogen, it was first decided to attempt to purify the commercial pepsinogen. Liener in 1950 (81) and Ruth and Perlmann in 1963 (39) have reported the purification of crude pepsinogen on DEAE-cellulose columns. Two roughly identical peaks were obtained. The potential pepsin activity was found to be associated with the second peak only. No yield of pure pepsinogen, based on the amount of crude pepsinogen used, was reported. In our work we have used Sephadex G-25; this compound has been used extensively in the last few years for the separation of proteins from materials of low molecular weight. The elution of protein and peptides from the column was followed by measuring the U.V. absorption at 230 m $\mu$  of the eluate and by paper chromatography. The fraction containing protein or smaller contaminants were lyophilized and the weight of the dry residues determined. The results are shown in Figure 1. The U.V. absorbing

Figure 1. Purification of pepsinogen on Sephadex G-25



material was found to be located mainly in one sharp peak, covering fractions 15 to 27; those fractions were also shown by paper chromatography to contain only one component; this unique component did not move at all on the paper chromatogram. As far as the recovery is concerned, it can be seen on Figure 1 that only 40 to 45 per cent of the original material was present in the pure fractions. This yield is relatively low, it is possible that the separation of pepsinogen from the impurities could have been improved by devoting more time to this problem. On the other hand, this purification process had the advantages of being a fast and simple method to produce very pure pepsinogen. It was therefore decided to use it without further improvements and to concentrate our efforts on the study of the activation process.

## (2) Activation of pepsinogen

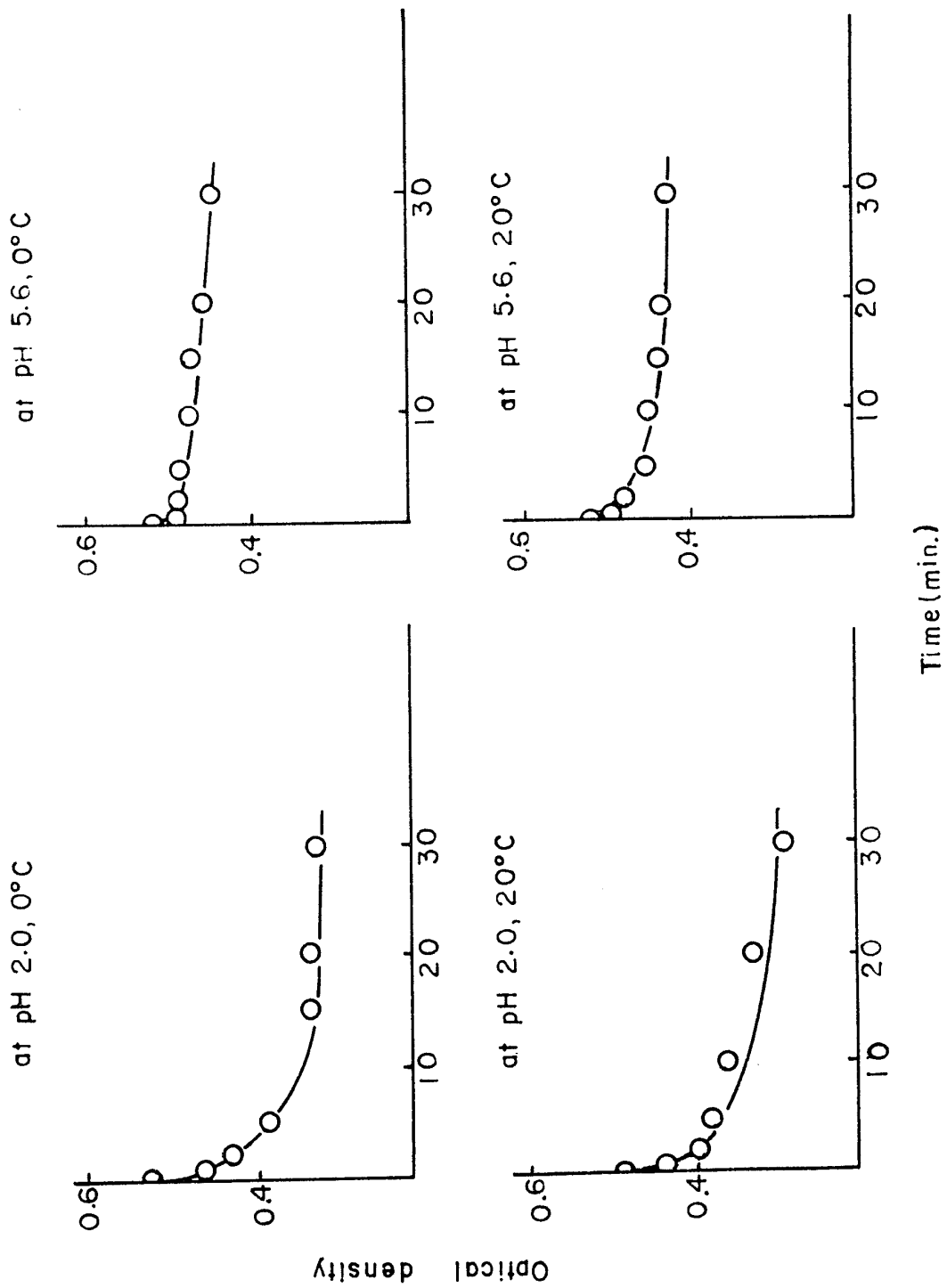
At pH values less than 6, pepsinogen is converted to pepsin by an autocatalytic reaction, the conversion is speeded by the addition of pepsin or by increasing the concentration of pepsinogen, the reaction has a maximum rate near the pH optimum of pepsin (pH 1.8), and the reaction is also catalyzed by hydrogen ions. In the present work, a rather high pepsinogen concentration was used (6.6 mg./ml.); this concentration was used for two reasons: firstly, in order to simplify the extraction procedure, the volume of the solutions had to be kept as small as possible; secondly, in order to get enough dinitrophenyl-peptides (DNP-peptides) to work with, large quantities of pepsinogen had to be used. The activation was first studied at pH 2.0 and at room temperature (20°C);

the process was found to be very fast (Figure 2). When the temperature was lowered near 0°C, the rate of the activation process was decreased a little, especially in the first two minutes of the reaction (Figure 2), but we did not obtain the big decrease in rate that we were hoping for. It should be pointed out that these curves (Figure 2) give the rate of disappearance of pepsinogen from the reaction mixture rather than the rate of appearance of pepsin. Pepsin activity measurements with the hemoglobin method are carried out under conditions which would normally activate pepsinogen rapidly, therefore the newly formed pepsin had to be first denatured at alkaline pH values and then the remaining pepsinogen activated at pH 2.0 and its activity determined by reaction with hemoglobin (23).

The curves in Figure 2-A were obtained from the data presented in Figure 2. The differences between the quantity of pepsinogen present at time zero and the pepsinogen remaining in the activation mixture after 1/2, 2, 5, 10, 20, and 30 minutes were converted into pepsin units according to the method of Northrop, Kunitz, and Herriott (8), these curves therefore give the rate of appearance of the newly formed pepsin in the activation mixture.

The activation process was also studied at pH 5.6. It is known that above pH 5.4 pepsin forms an inactive complex with one of the peptides liberated during the activation, this peptide is called the pepsin inhibitor. By running the activation at pH 5.6, it was hoped that the newly formed pepsin would be very rapidly inactivated by the

Figure 2. Activation of pepsinogen

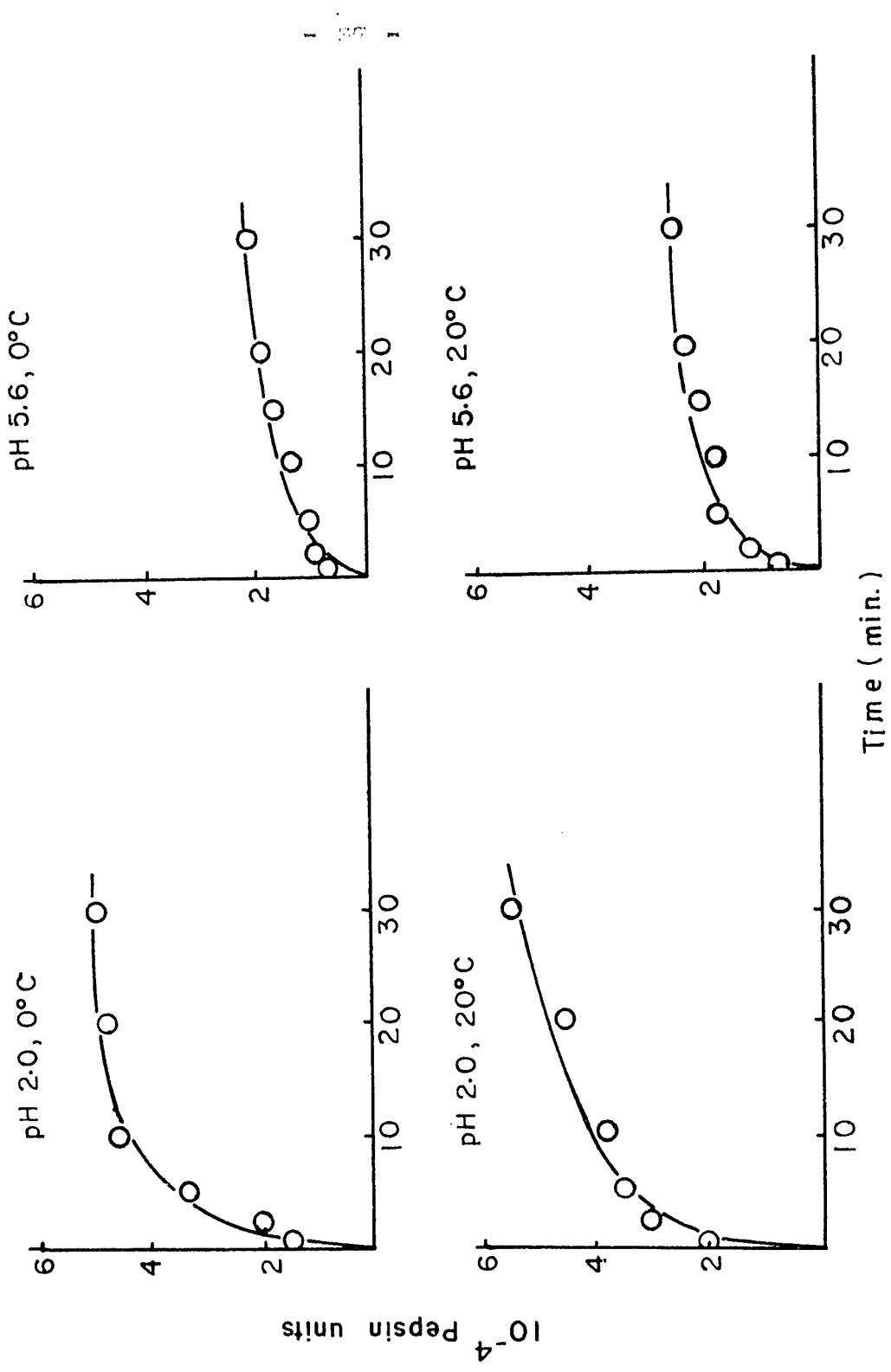


Division of Fractions

	1 min.	$\frac{1}{2}$ min.	1 min.	2 min.	3 min.	4 min.	5 min.	10 min.	15 min.	20 min.	30 min.
$\frac{1}{2} \div \frac{1}{3}$	$\frac{1}{2} \times \frac{3}{1}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$
$\frac{1}{3} \div \frac{1}{4}$	$\frac{1}{3} \times \frac{4}{1}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$	$\frac{4}{3}$
$\frac{1}{4} \div \frac{1}{5}$	$\frac{1}{4} \times \frac{5}{1}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$	$\frac{5}{4}$
$\frac{1}{5} \div \frac{1}{6}$	$\frac{1}{5} \times \frac{6}{1}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$	$\frac{6}{5}$

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Figure 2-A Activation of pepsinogen

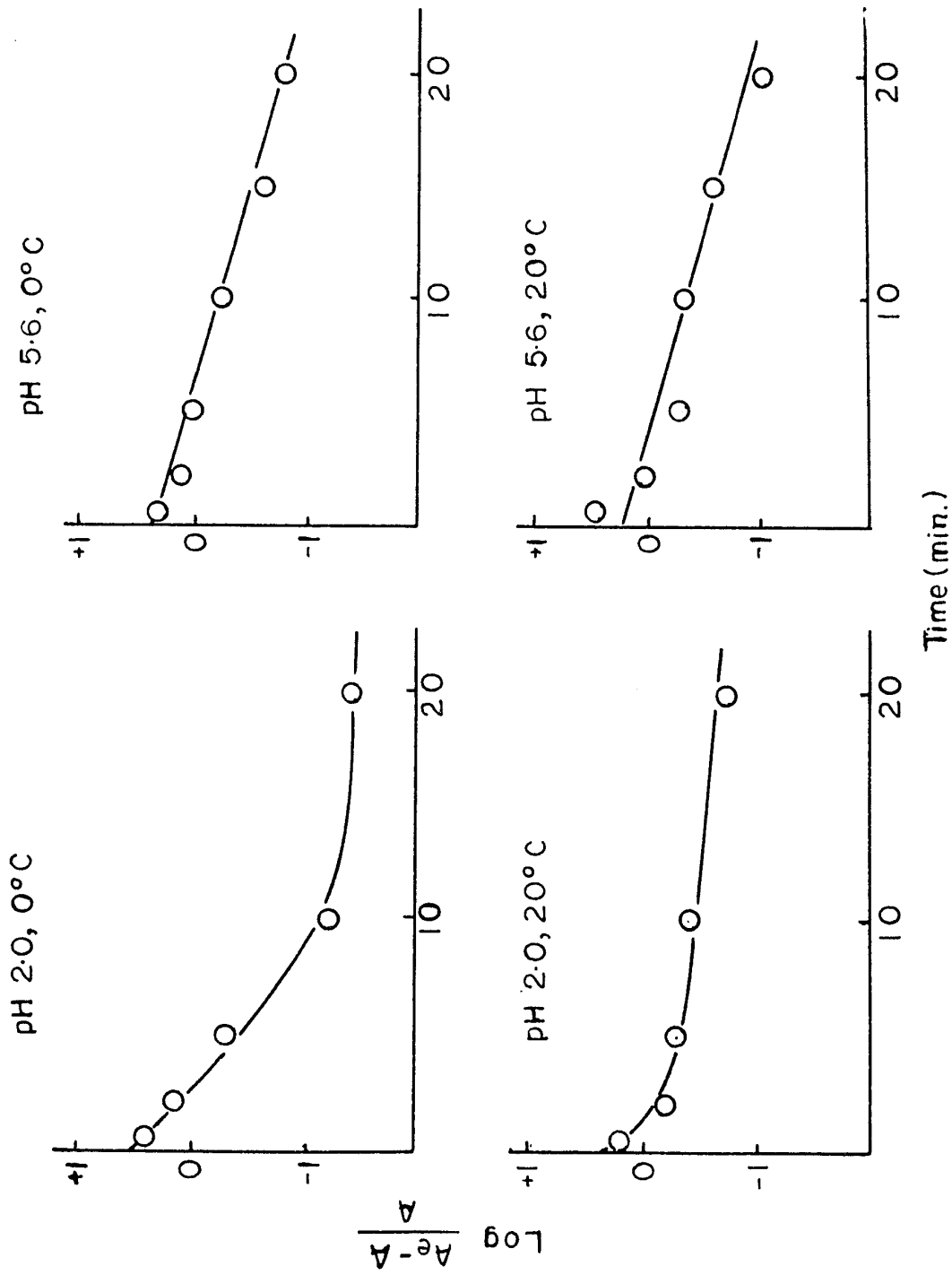


peptide inhibitor, and that this inactivation would prevent any pepsin fragmentation due to autolysis. Activation at pH 5.6 was also run at two different temperatures, 20°C and 0°C (Figure 2 and 2-A). At pH 5.6 and 20°C, it is quite evident from the curves presented that the activation process is slow and when the temperature is decreased to 0°C the process is still slower. It can be seen also that after 30 minutes the amount of pepsinogen remaining in the pH 5.6 activation mixture is much greater than the amount remaining in the pH 2.0 activation mixture. In the pH 2.0 activation mixtures 57% and 61% of the original pepsinogen was left after 30 minutes at room temperature or at 0°C. In the pH 5.6 activation mixture on the other hand, 82% and 84% of the original pepsinogen was still present after 30 minutes at room temperature or at 0°C. In other words, after 30 minutes at pH 2.0 and 20°C, 43% of the pepsin potential activity had been liberated, and after 30 minutes at pH 2.0 and 0°C only 39%. At pH 5.6 only 18% and 16% of the pepsin potential activity had been liberated after 30 minutes at 20°C and 0°C respectively. The 40% yield obtained at pH 2.0 is a little surprising, because it is generally assumed that pepsinogen is fully activated by standing a few minutes at pH 2. After 10 minutes at pH 2.0 and 0°C, Herriott (28) obtained conversion of about 90% of its pepsinogen into pepsin; at pH 3.0 and 0°C, he got a 45 to 50% yield after 20 minutes (71). The yields obtained at pH 5.6 are more in line with the results presented by Herriott (28) for activation at pH 4.6 and 25°C. But in all four cases, the curves that we have obtained are not representative of an autocata-

lytic process as was the curve obtained by Herriott for activation at pH 4.6 (23). On the contrary, in our activation systems the rate is very high in the first few minutes of activation, and much higher at pH 2.0 than at pH 5.6. This would imply that hydrogen ions are the main catalytic agents. This has been already noticed by Herriott (23) who concluded that the reaction is autocatalytic above pH 4.0, but that in solution more acid than pH 4.0, it deviates from the simple autocatalytic process. In fact only the curve obtained for activation at pH 5.6 and 0°C (Figure 2-A) could represent the beginning of the S-shape curve, typical of the autocatalytic activation. Herriott (72) plotted his results as the log of  $(A_0 - A)/A$  against the time, where  $A_0$  is the final pepsin activity and  $A$  is the activity at time  $t$ . This method of plotting gives a straight line showing that the reactions are autocatalytic; the slope of the line is called the autocatalytic constant  $K$ . The expression  $\log \frac{A_0 - A}{A}$  is derived from the simple autocatalytic equation  $dA/dt = KA(A_0 - A)$ . We have plotted our results in accordance with the autocatalytic equation, i.e.  $\log \frac{A_0 - A}{A}$  against time (Figure 2-B). The pH 5.6 and 0°C and the pH 5.6 and 20°C activation mixtures give good straight lines, the autocatalytic constants are 0.061 and 0.060 respectively. As far as the pH 2.0 activation is concerned, it is quite evident from the graphs presented in Figure 2-B that the reaction is more complex than a simple autocatalytic activation process.

In all our experiments the activation process was stopped after 30 minutes. Our intentions were to study the first chemical events

Figure 2-B Autocatalytic activation of pepsinogen



occurring during activation, and if possible, to characterize the first peptide liberated from pepsinogen, therefore we tried to keep the activation mixture as simple as possible by using short time periods.

### (3) Activation of DNP-pepsinogen

Recently Lokshina and Orekhovich (76) studied the activation of DNP-pepsinogen. They claimed that the acidification of a DNP-pepsinogen solution for three minutes at room temperature is accompanied by the release of a DNP-peptide. We have tried to repeat their results, but unfortunately without success. We found out that as soon as the pH of the DNP-pepsinogen solution is lowered below 5, the DNP-protein is precipitated out of the solution leaving a colorless solution. It seems therefore doubtful that DNP-pepsinogen can be activated at all at pH values lower than 5.

### (4) Fractionation of the activation mixtures

We first attempted to fractionate the pepsinogen activation mixtures using conventional paper chromatography and paper electrophoresis method. The whole activation mixtures or the TCA supernatants were used in these fractionation attempts, but no clear separation of the components could be obtained. Ion exchange resins and Sephadex G-25 were also tested, but here again we did not get good separation of all the components. The method finally used consisted in treating the activation mixture with fluorodinitrobenzene in  $\text{NaHCO}_3$ . After acidification of the reaction mixtures and extraction with ethyl acetate, we obtained three

fractions: 1. the insoluble DNP-proteins; 2. the aqueous layer containing big DNP-peptides insoluble in organic solvents; 3. the ethyl acetate layer containing dinitrophenol, dinitroaniline, soluble DNP-peptides, and DNP-amino acids.

Not much work was done on the insoluble DNP-proteins. This is a mixture of DNP-pepsinogen and DNP-pepsin.

The water and ethyl acetate layers were fractionated by paper chromatography using the Blackburn and Lowther's method (79). In the case of the water layer a 48 hour run was used and a very good resolution of 4 to 5 components was obtained, those components were designated  $W_1$ ,  $W_2$ ,  $W_3$ ,  $W_4$ , and  $W_5$  (Table 1). All four activation mixtures were found to contain  $W_1$ ,  $W_2$ ,  $W_3$  and  $W_5$ ,  $W_4$  was absent from the pH 2.0, 20°C activation mixture. As far as  $W_5$  (the fastest DNP component) is concerned, the distance travelled was found to vary considerably.

The ethyl acetate layer was more difficult to fractionate, a shorter run (24 hrs.) had to be used in order to prevent the fastest component from running off the paper. In addition the yellow bands on the paper were not very sharp.  $E_1$ , the yellow band remaining at the origin, the dinitrophenol band, and the dinitroaniline band were easy to recognize.  $E_2$ ,  $E_3$ , and  $E_5$  were not well defined yellow bands; they were yellow zones.  $E_2$  and  $E_3$  were between the origin and the dinitrophenol band ( $E_4$ ), and  $E_5$  was situated between the dinitrophenol and the dinitroaniline bands.

Table 1. Distances travelled by DNP-peptides from activation mixtures on Whatman No. 3 MM paper in tertiary amyl alcohol-phthalate buffer pH 6.8 system

Fractions	pH 2.0		pH 5.6	
	0°C.	20°C.	0°C.	20°C.
	cm./48 hrs.	cm./48 hrs.	cm./48 hrs.	cm./48 hrs.
Water phase: W <sub>1</sub>	0	0	0	0
W <sub>2</sub>	5.0	4.5	3.8	5.5
W <sub>3</sub>	7.7	10.5	8.2	7.7
W <sub>4</sub>	10.2	-	14.3	9.5
W <sub>5</sub>	15.3	28.0	26.7	13.4
	cm./24 hrs.	cm./24 hrs.	cm./48 hrs.	cm./24 hrs.
EtoAc phase: E <sub>1</sub>	0	0	0	0
E <sub>2</sub>	2.6	4.0	9.5	2.0
E <sub>3</sub>	6.4	6.0	14.0	10.3
E <sub>5</sub>	20.4	-	-	-

It should be pointed out also that when purified pepsinogen or crystalline pepsin are treated with FDNB, the aqueous layer contains only  $W_1$ , and the ethyl acetate layer only  $E_1$ , dinitrophenol, and dinitroaniline. It is therefore evident that the DNP-derivatives except  $W_1$  and  $E_1$  present in the water and ethyl acetate layers, obtained from the activation mixtures, were formed as a result of the transformation of pepsinogen into active pepsin. The rate of production of these DNP-peptides was estimated by determining the optical density of the DNP-peptide solutions (after extraction of the DNP-derivatives from the paper) in the Beckman DU spectrophotometer for four different periods of activation. The curves showing the increase with time of each component are given in Figs. 3, 4, 5 and 6.

If we look first at the quantitative aspect of those curves, it is evident that activation at pH 2.0 produces much more DNP-derivatives than activation at pH 5.6, 10 to 15 times more. This is in line with the results presented above (Fig. 2 and 2-A) that activation at pH 2.0 produces more active pepsin within 30 minutes than activation at pH 5.6. In three cases at least Fig. 3, 4, and 5, it is also evident that  $W_2$  is the component produced at the highest rate.  $W_1$  which is not moving at all on the paper chromatogram (Table 1), contains all the amino acids (Table 4), has an isoleucine or a valine N-terminal residue (Table 2) and an alanine C-terminal residue (Table 3), and is also present in the pepsinogen-FDNB and the pepsin-FDNB reaction mixtures.

Figure 3. Liberation of peptides from pepsinogen during activation at pH 2.0 and 20° C

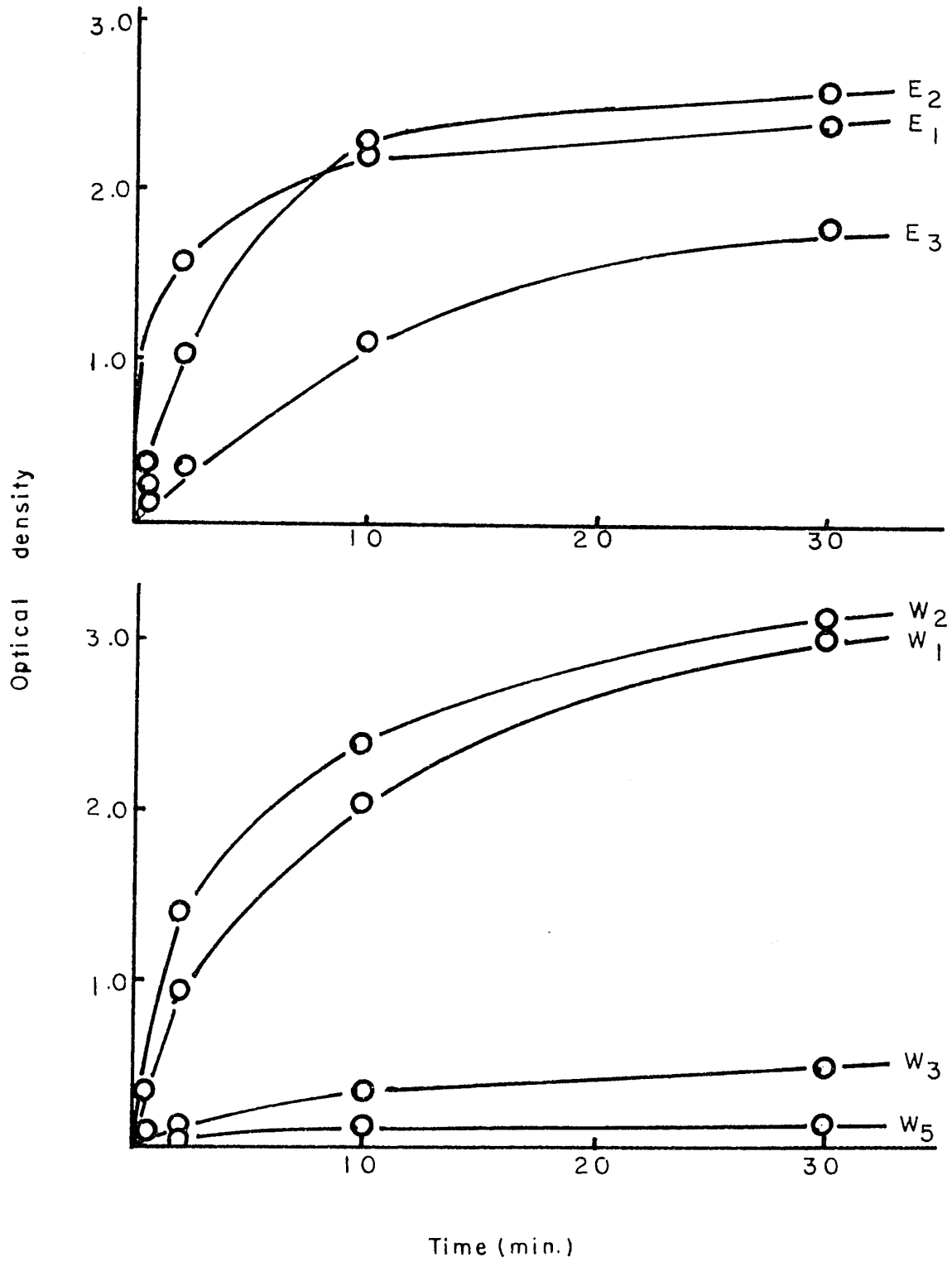


Figure 4. Liberation of peptides from pepsinogen during activation at pH 2.0 and 0°C

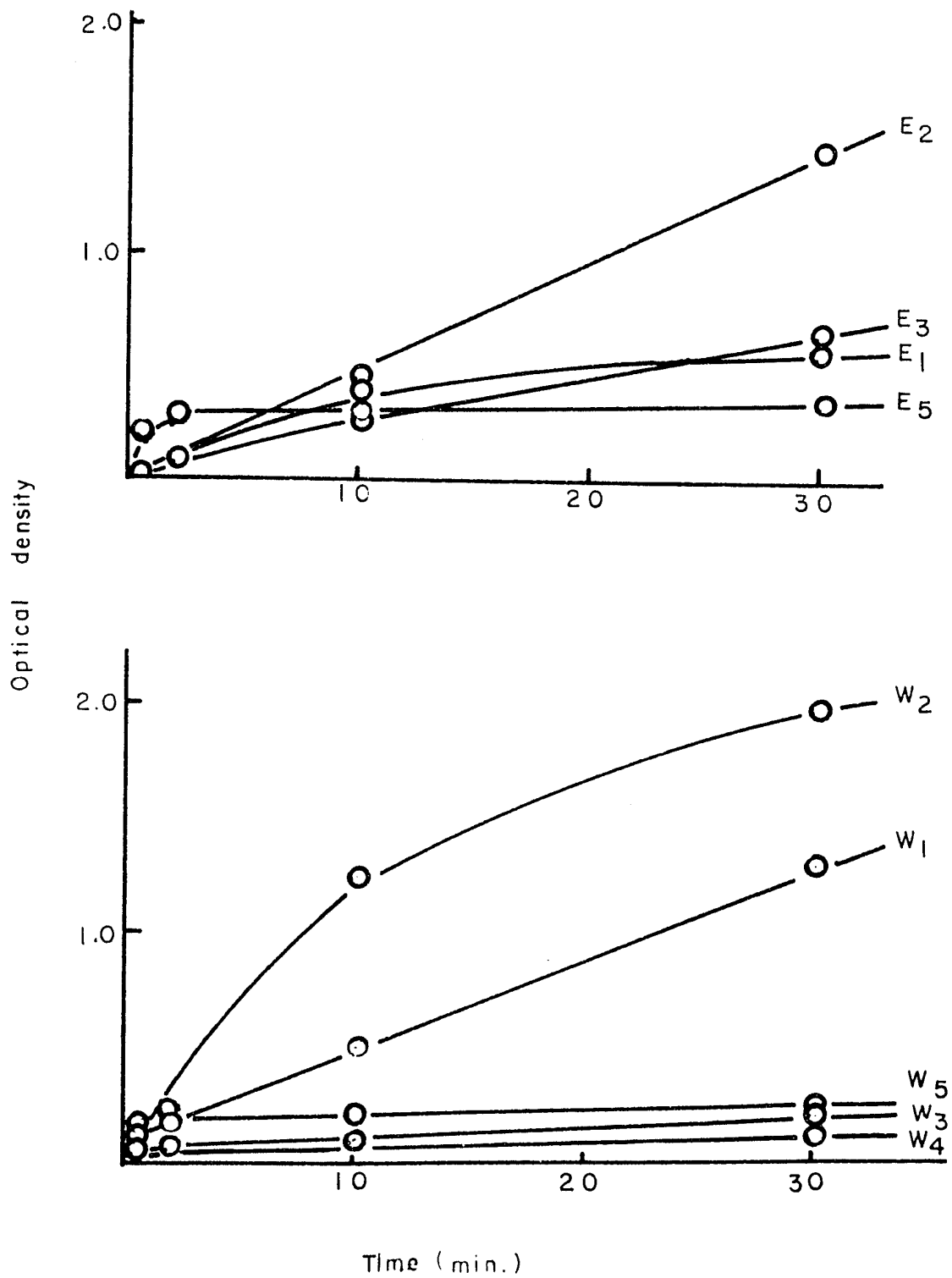


Figure 5. Liberation of peptides from pepsinogen during activation at pH 5.6 and 20°C

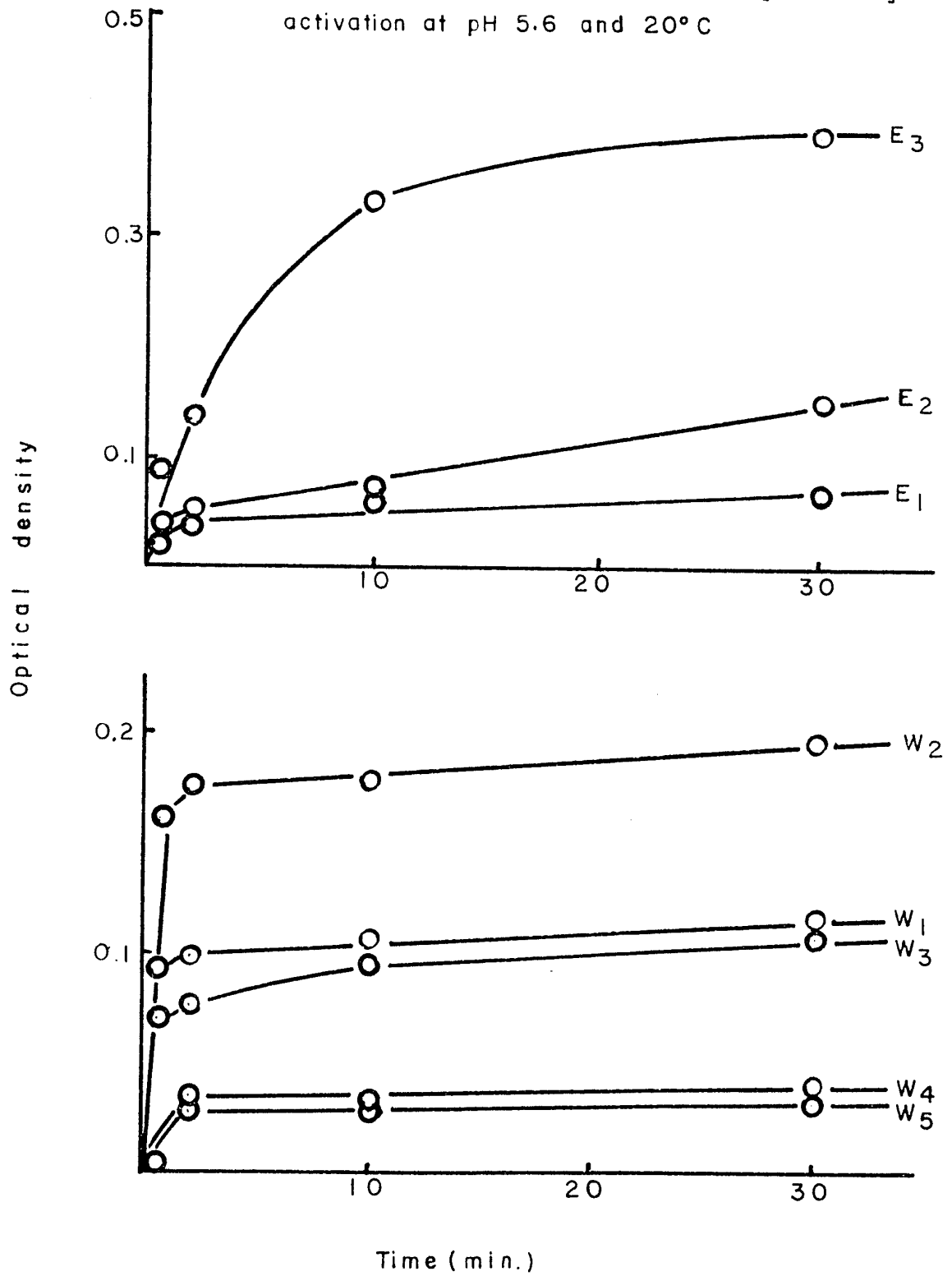


Figure 6. Liberation of peptides from pepsinogen during activation at pH 5.6 and 0° C

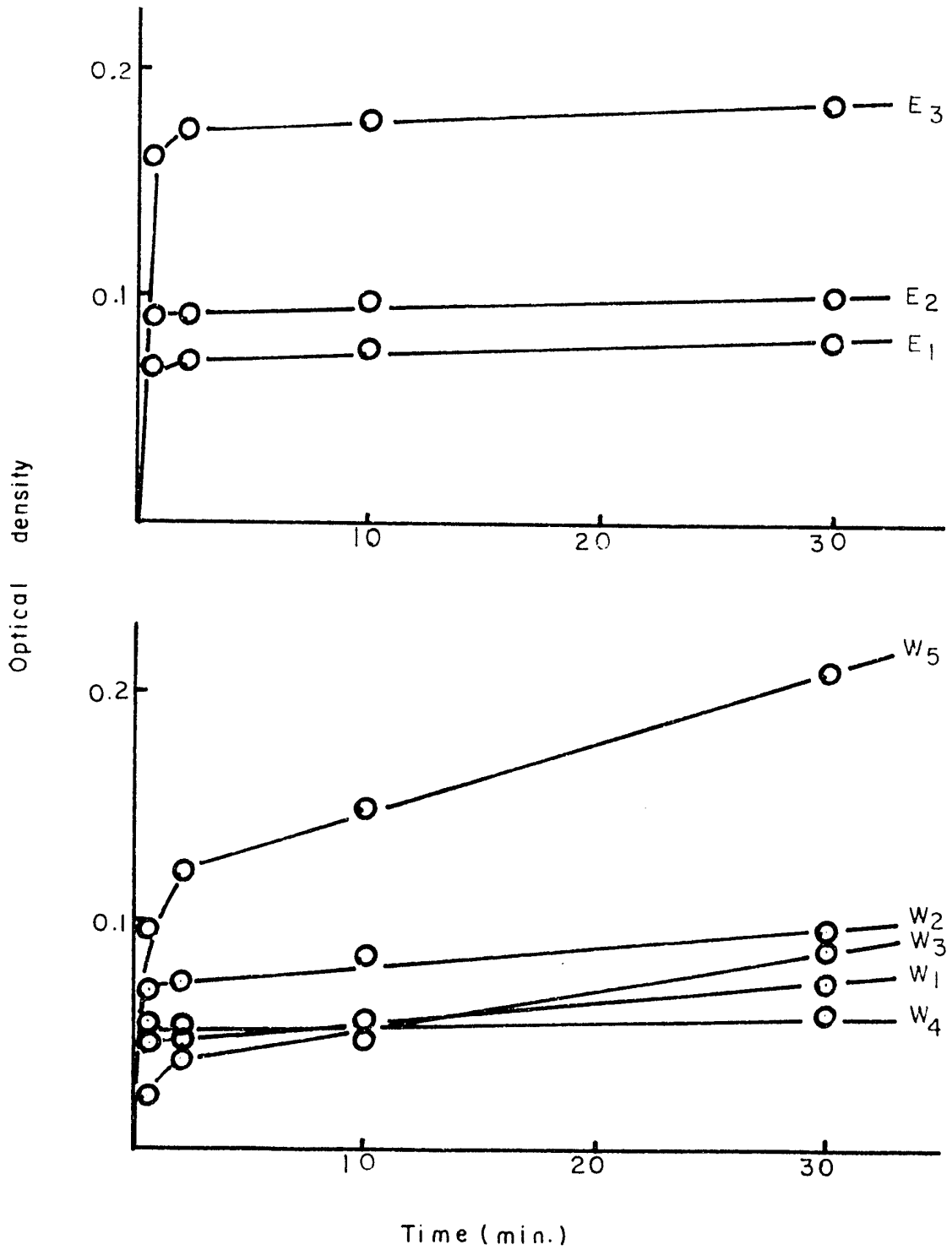


Table 2. N-terminal groups of peptides isolated from the activation mixtures

Peptides	N-terminal residues		
	pH 2.0, 0°C	pH 2.0, 20°C	pH 5.6, 20°C
W <sub>1</sub>	-	Ileu	Val
W <sub>2</sub>	-	Ieu	Val
W <sub>3</sub>	Ieu	Ieu	Ieu
W <sub>4</sub>	Val	-	Val
W <sub>5</sub>	-	Ieu	Val
E <sub>1</sub>	-	Ileu	Ieu
E <sub>2</sub>	Ieu	Ieu	Ieu
E <sub>3</sub>	Ieu	Ieu	Val
E <sub>5</sub>	Val	-	-

Table 3. C-terminal residues of the peptides isolated from the activation mixtures

Peptides	C-terminal residues	
	pH 2.0, 0°C	pH 2.0, 20°C
W <sub>1</sub>	Ala-leu-(val-phe-)	Ala-(leu-val-)
W <sub>2</sub>	Asp-leu-phe-	Asp-leu-phe-(gly-val-)
W <sub>3</sub>	His-Ser-leu-	His-ser-(leu-arg-gly-)
W <sub>4</sub>	Leu-val-ser-	-
W <sub>5</sub>	Leu-(asp-val-)	Leu-asp-val-
E <sub>1</sub>	Ala-(leu-val-)	Ala-leu-val-
E <sub>2</sub>	His-ser-leu-	His-ser-
E <sub>3</sub>	Leu-asp-	Leu-asp-
E <sub>5</sub>	Leu-val-	-

Table 4. Amino acid composition of the peptides isolated from the activation mixtures

a. pH 2.0, 20°C

Peptides	N-terminal	amino acid composition (excluding N-terminal residue)	C-terminal
W <sub>1</sub>	Ileu	Cys (1), Asp (4), Ser (4), Glu (2), Gly (2), Thr (2), Ala (2), Pro (2), Lys (2), His (1), Arg (1), Val (3), Phe (1), Leu and Ileu (4), Tyr (1), Meth (1).	-val-leu-ala
W <sub>2</sub>	Leu	Asp (3), Ser (3), Glu (3), Gly (2), Thr (2), Ala (2), Pro (2), Lys (3), His (1), Arg (1), Val (2), Phe (1), Leu and Ileu (4), Tyr (1).	-val-gly-phe-leu-asp
W <sub>3</sub>	Leu	Asp (3), Ser (2), Glu (3), Gly (2), Thr (1), Ala (2), Pro (2), Lys (3), His (2), Arg (1), Val (1), Phe (1), Leu and Ileu (2), Tyr (1).	-gly-arg-leu-ser-his
W <sub>5</sub>	Leu	Asp (2), Ser (1), Glu (2), Gly (1), Ala (1), Pro (2), Lys (2), Val (2), Phe (1), Leu and Ileu (3).	-val-asp-leu
E <sub>1</sub>	Ileu	Cys (1), Asp (4), Ser (4), Glu (2), Gly (2), Thr (2), Ala (2), Pro (2), Lys (2), His (1), Arg (1), Val (3), Phe (1), Leu and Ileu (3), Tyr (1), Meth (1).	-val-leu-ala
E <sub>2</sub>	Leu	Asp (3), Ser (2), Glu (3), Gly (2), Thr (1), Ala (1), Pro (2), Lys (3), His (2), Arg (1), Val (1), Phe (1), Leu and Ileu (2).	-ser-his
E <sub>3</sub>	Leu	Asp (2), Ser (1), Glu (2), Gly (1), Ala (1), Pro (1), Lys (2), Val (2), Phe (1), Leu and Ileu (3).	-asp-leu

b. pH 2.0, 0°C

Peptides	N-terminal	amino acid composition (excluding N-terminal residue)	C-terminal
W <sub>3</sub>	Leu	Asp (2), Ser (3), Glu (2), Gly (2), Thr (1), Ala (3), Pro (2), Lys (2), His (1), Arg (1), Val (2), Phe (1), Tyr (1), Leu and Ileu (3).	-leu-ser-his
W <sub>4</sub>	Val	Asp (2), Ser (3), Glu (3), Gly (2), Thr (2), Ala (3), Lys (2), His (1), Arg (1), Val (2), Phe (1), Tyr (1), Leu and Ileu (3).	-ser-val-leu
E <sub>2</sub>	Leu	Asp (1), Ser (2), Glu (3), Gly (1), Thr (1), Ala (3), Lys (1), Pro (1), His (1), Arg (1), Val (2), Phe (1), Tyr (1), Leu and Ileu (3).	-leu-ser-his
E <sub>3</sub>	Leu	Asp (2), Ser (2), Glu (2), Gly (2), Thr (2), Ala (2), Pro (1), Lys (1), Val (1), Phe (1), Tyr (1), Leu and Ileu (2).	-asp-leu
E <sub>5</sub>	val	Asp (1), Ser (2), Glu (1), Gly (1), Ala (2), Lys (1), Val (2), Phe (1), Tyr (1), Leu and Ileu (2).	-val-leu

It is probably a mixture of DNP-pepsinogen and DNP-pepsin which are very slightly soluble in water. The increase with time of  $W_1$  would indicate that DNP-pepsin is more soluble than the DNP-pepsinogen.

As far as  $W_2$  is concerned, it is evident that it is the major component produced during activation at pH 2.0. The initial rate of  $W_2$  production corresponds to the rate of pepsin production in all four cases studied. At pH 5.6 where the pepsin formation reaches a plateau very rapidly, the  $W_2$  formation is also very fast in the first few minutes and then becomes almost negligible. In most cases also the production of  $W_2$  follows pretty well the production of  $W_1$ . If we assume that  $W_1$  is mostly DNP-pepsin, then those curves indicate that  $W_2$  is the most important peptide released from pepsinogen when it is transformed into pepsin.

Our results indicate also that  $W_2$  is not the only peptide liberated during activation, at least three other big peptides,  $W_3$ ,  $W_4$ , and  $W_5$ , are also formed. The amount of these peptides produced is very small as compared to  $W_1$  and  $W_2$ . At pH 2.0 there is a small increase during the first 2 minutes and then the quantity of these peptides remains almost constant. At pH 5.6 the situation is a little different. At room temperature  $W_3$  production follows  $W_1$  and  $W_2$  production while  $W_4$  and  $W_5$  do not increase any more after 2 minutes. At 0°C the situation is reversed,  $W_5$  becomes the most important peptide formed, and even after 30 minutes the quantity of  $W_5$  produced is still increasing.  $W_3$  shows the same type of curve but on a much lower level.  $W_4$  does not increase any more after the first minute. It should be remembered that at pH 5.6 and 0°C the

pepsin production reaches a plateau very rapidly (Fig. 2-A).

The peptides  $W_3$ ,  $W_4$ , and  $W_5$  could be considered as:

1. peptides liberated from pepsinogen during its conversion into active pepsin.
2. peptides produced by the action of the newly formed pepsin on  $W_2$ .
3. peptides produced by autolysis of pepsin.

If these peptides were formed by proteolysis, action of pepsin on  $W_2$  or on itself, one would expect that the quantity of these peptides present in the activation mixture would increase with time. But this is not the case (Fig. 3, 4, 5, and 6) for most of them. On the contrary the quantity increases only in the first few minutes of activation, and therefore it seems more probable that their formation is associated with the production of active pepsin. Even in the case of  $W_5$  at pH 5.6 and 0°C (Fig. 6), it should be remembered that after 30 minutes the total quantity of  $W_5$  is not larger than the quantity of  $W_5$  present in the pH 2.0 activation mixtures (Fig. 3 and 4) after 30 minutes. It is however much higher than the amount of  $W_5$  present in the pH 5.6 and 20°C activation mixture (Fig. 5).

It is reasonable to assume that the liberation of  $W_2$ ,  $W_3$ ,  $W_4$ , and  $W_5$  involves in each case the cleavage of one peptide bond. If this is right, then one must assume that more than one species of active pepsin is formed by removal of a given peptide from the N-terminal end of pepsinogen. The  $W_2$  pepsin, the one formed by removal of the  $W_2$  peptide from pepsinogen, would be the most important species of active pepsin. The  $W_3$ ,  $W_4$ , and  $W_5$  pepsins would be less important. At pH 5.6

and 0°C the W<sub>5</sub> pepsin would be the most important species formed. The amount of each peptide formed would therefore depend on the susceptibility of a given peptide bond to acid or peptic hydrolysis. It is quite evident that the peptide bond cleaved when W<sub>2</sub> is liberated is very susceptible to acid hydrolysis, and that at pH 2.0 the autocatalytic activation process is not important. The peptide bonds involved in liberation of W<sub>3</sub>, W<sub>4</sub> and W<sub>5</sub> are less rapidly cleaved in very acid solution. The least susceptible would be the W<sub>4</sub> peptide bond; this peptide is not formed in any measurable amount at pH 2.0 and 20°C. The peptide bonds W<sub>2</sub>, W<sub>3</sub>, and W<sub>5</sub> being probably cleaved too rapidly. At pH 2.0 and 0°C, where it is evident that the speed of cleavage of the peptide bonds is decreased, then a little W<sub>4</sub> peptide bond is also cleaved.

At pH 5.6 it would seem that the hydrogen ion catalysis is less important, because the production of peptides stops after a few minutes as well as the pepsin production. If we assume that at this pH the autocatalytic activation is important, then the formation of the pepsin-inhibitor complex would prevent any big autocatalytic effect after a few minutes. Also at this pH the susceptibility of the peptide bonds to hydrolysis is slightly changed with temperature. At 0°C the hydrolysis of the W<sub>2</sub> and W<sub>3</sub> peptide bonds seems to be slower than that at 20°C, and this results in an increased production of W<sub>4</sub> and W<sub>5</sub> as more of these bonds are available. It seems also that at pH 5.6 and 0°C the formation of the pepsin-inhibitor complex is slower than that at 20°C. After 30 minutes, the amount of W<sub>3</sub> and W<sub>5</sub> is still increasing.

If this discussion is right, then we must conclude that the W<sub>2</sub> peptide bond is the one situated farthest away from the N-terminal residue of pepsinogen. Its cleavage would liberate the biggest peptide, and also would prevent the immediate cleavage of the W<sub>3</sub>, W<sub>4</sub>, and W<sub>5</sub> peptide bonds which would be closer to the N-terminal residue of pepsinogen, W<sub>5</sub> being the closest of them all.

As it was stated before the importance of the ethyl acetate soluble components is more difficult to assess. From the amino acid analysis, N-terminal and C-terminal residues determination, it would seem that the zones E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub> contain also the water soluble components W<sub>1</sub> to W<sub>5</sub>. It is very likely that these DNP-derivatives are slightly soluble in ethyl acetate. Because of the shorter running time used in developing the chromatogram, it could be expected that the ethyl acetate bands corresponding to W<sub>1</sub>, W<sub>2</sub>, W<sub>4</sub>, W<sub>4</sub> and W<sub>5</sub> would be situated between the origin and the dinitrophenol band, hence in E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub>. E<sub>5</sub> would contain only DNP derivatives of very small peptides and free amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine and proline. E<sub>1</sub> is the yellow component remaining at the origin, it is probably identical with W<sub>1</sub>, DNP-pepsinogen and DNP-pepsin. W<sub>2</sub> could also be present in E<sub>1</sub>, as W<sub>2</sub> is running very slowly. E<sub>2</sub>, the first yellow zone moving very slowly in tertiary amyl alcohol, probably contains W<sub>2</sub> and W<sub>3</sub>; while E<sub>3</sub>, the second yellow zone, could be a mixture of W<sub>4</sub> and W<sub>5</sub>. At pH 2.0, E<sub>2</sub> is the component produced at the highest rate, while at pH 5.6 it is less important than E<sub>3</sub>. Furthermore in most cases

the production of E<sub>2</sub> corresponds to the production of E<sub>1</sub>, the pH 2.0 and 0°C activation mixture being the only exception. In the last case after 10 minutes E<sub>2</sub> goes on increasing while E<sub>1</sub> remains constant. As far as E<sub>3</sub> is concerned, this zone is important in the pH 2.0 and 20°C activation mixture. The large quantity of E<sub>3</sub> could be a result of the greater solubility of W<sub>4</sub> and W<sub>5</sub> in ethyl acetate. If this is right, then the role of W<sub>4</sub> and W<sub>5</sub> in the activation of pepsinogen could be more important than it was first believed after the examination of the water phase.

At pH 5.6 the situation is reversed. E<sub>3</sub> becomes the most important component of the ethyl acetate phase. On a quantitative basis, it would mean that at pH 5.6, at 20°C as well as 0°C, the peptides W<sub>4</sub> and W<sub>5</sub> would be produced in larger quantity than W<sub>2</sub> and W<sub>3</sub>, therefore at pH 5.6 the activation of pepsinogen would produce a mixture of many species of pepsin in almost equal amount. These different pepsins would be produced by autocatalysis and by hydrogen ion catalysis. On the other hand, at pH 2.0 the activation mixture would contain one main pepsin species (the W<sub>2</sub> pepsin) and smaller amounts of the other species. Hydrogen ion catalysis would be the main process involved in the activation. The W<sub>2</sub> peptide bond would be the most susceptible to acid hydrolysis.

(5) Analysis of the N-terminal residues of the DNP-derivatives isolated from the activation mixtures

The N-terminal residues of the DNP-derivatives isolated from the activation mixtures were identified by paper chromatography after

acid hydrolysis of the derivatives. Positive identification of the N-terminal residues was not easy in many cases due to the small amounts of DNP-derivatives involved. Table 2 gives the list of the N-terminal residues which were identified. The main difference between pH 2.0 and pH 5.6 activation is that in many cases a valine replaces leucine as the N-terminal residue ( $W_1$ ,  $W_2$ ,  $W_5$ , and  $E_3$ ).  $W_1$  and  $E_1$  from pH 2.0 activation mixture have an isoleucine N-terminal residues, this is the same N-terminal residue as pepsin. This seems to indicate that the main species of pepsin produced has an isoleucine a N-terminal. At pH 5.6 the main N-terminal residues identified were valine for  $W_1$  and leucine for  $E_1$ , this could mean that the other pepsin species produced have different N-terminal residues. Leucine and isoleucine have been reported as N-terminal residues of pepsin (34, 64).

It must be pointed out that in many cases especially in the case of the ethyl acetate soluble components more than one yellow spot was visible on the paper chromatograms, but the other spots were present in trace amounts only and it was not possible to identify them positively. These spots therefore were not included in Table 2, but this indicates that the ethyl acetate components were not as pure as the water components. Table 2 also shows a sort of specificity for the cleavage of peptide bonds in which the -NH- group is provided by leucine or isoleucine at pH 2.0 and leucine and valine at pH 5.6.

(6) Analysis of the C-terminal sequences of the DNP-derivatives isolated from the activation mixtures

The C-terminal sequences of the DNP-derivatives were studied with carboxypeptidase. The amino acids liberated were identified by paper chromatography. The rates of liberation are given for some of the DNP-derivatives in Figs. 7, 8, 9, and 10. In all cases the C-terminal residue is very easily identified. In many cases it is also possible to identify the second and the third residues from the end of the peptide chain. The C-terminal sequences identified are listed in Table 3. This table indicates that the main constituent of E<sub>2</sub> is W<sub>3</sub> and the main one of E<sub>3</sub> is W<sub>5</sub>. It is probable that during the 24 hour run used to fractionate the ethyl acetate layer, the W<sub>2</sub> peptide did not move enough from the origin to be seen as a distinct yellow band. This table also does not show any specificity as far as the amino acid providing the -CO- group to the peptide bond being cleaved is concerned. It must be remembered that pepsin has very broad specificity (78). The C-terminal sequence of W<sub>1</sub> and E<sub>1</sub> is the same as the one identified by other workers (68, 69) for pepsin and pepsinogen. This gives more weight to our previous assumption that W<sub>1</sub> was a mixture of DNP-pepsin and DNP-pepsinogen. Also if we assume that our previous discussion about the liberation of different peptides from the N-terminal end of pepsinogen is right, then when W<sub>2</sub> is liberated, an aspartyl-isoleucyl bond would be broken. When W<sub>3</sub> is liberated, a histidyl-valyl (or -leucyl) bond would be involved. The liberation of W<sub>4</sub> and W<sub>5</sub> would involve the cleavage of a

Figure 7. Action of carboxypeptidase on fractions  $W_1, W_2, W_3,$  and  $W_4$  from pH 2.0 activation mixture at 0°C

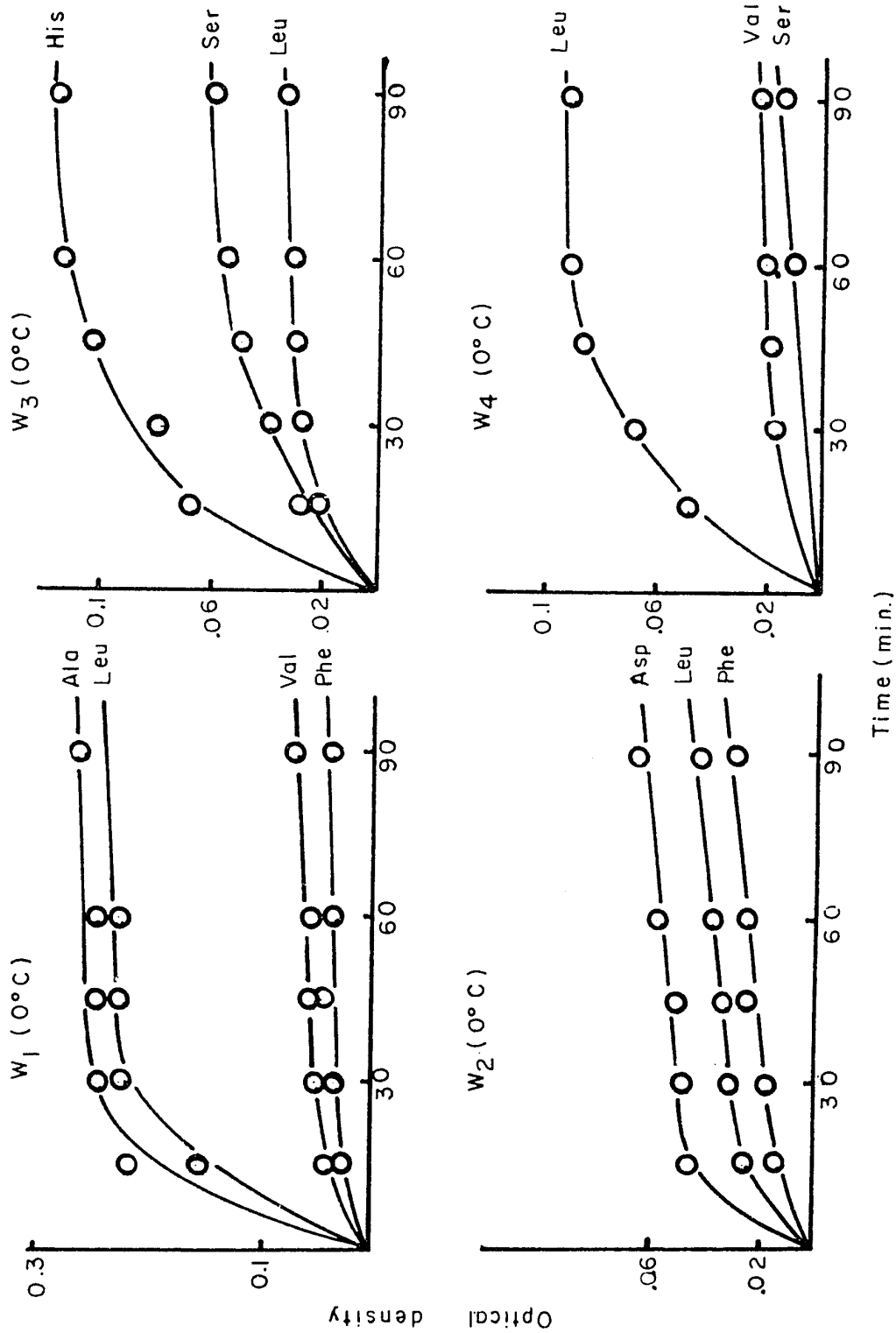


Figure 8. Action of carboxypeptidase on fractions W<sub>5</sub>, E<sub>1</sub>, E<sub>2</sub>, and E<sub>3</sub> from pH 2.0 activation mixture at 0°C

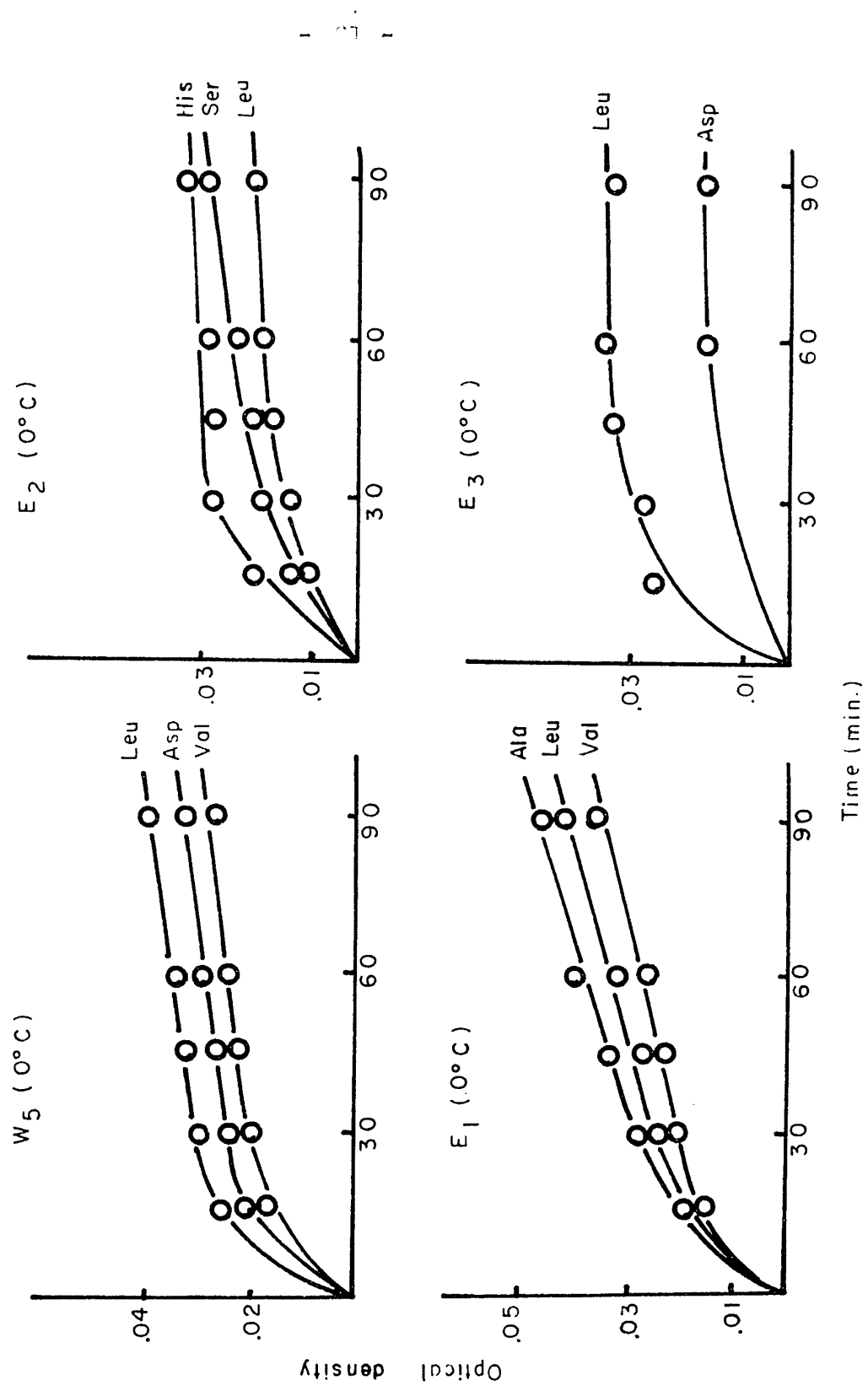


Figure 9. Action of carboxypeptidase on fraction E<sub>5</sub> from pH 2.0 activation mixture at 0° C and fractions W<sub>1</sub>, W<sub>2</sub>, W<sub>3</sub> from pH 2.0 activation mixture at 20° C

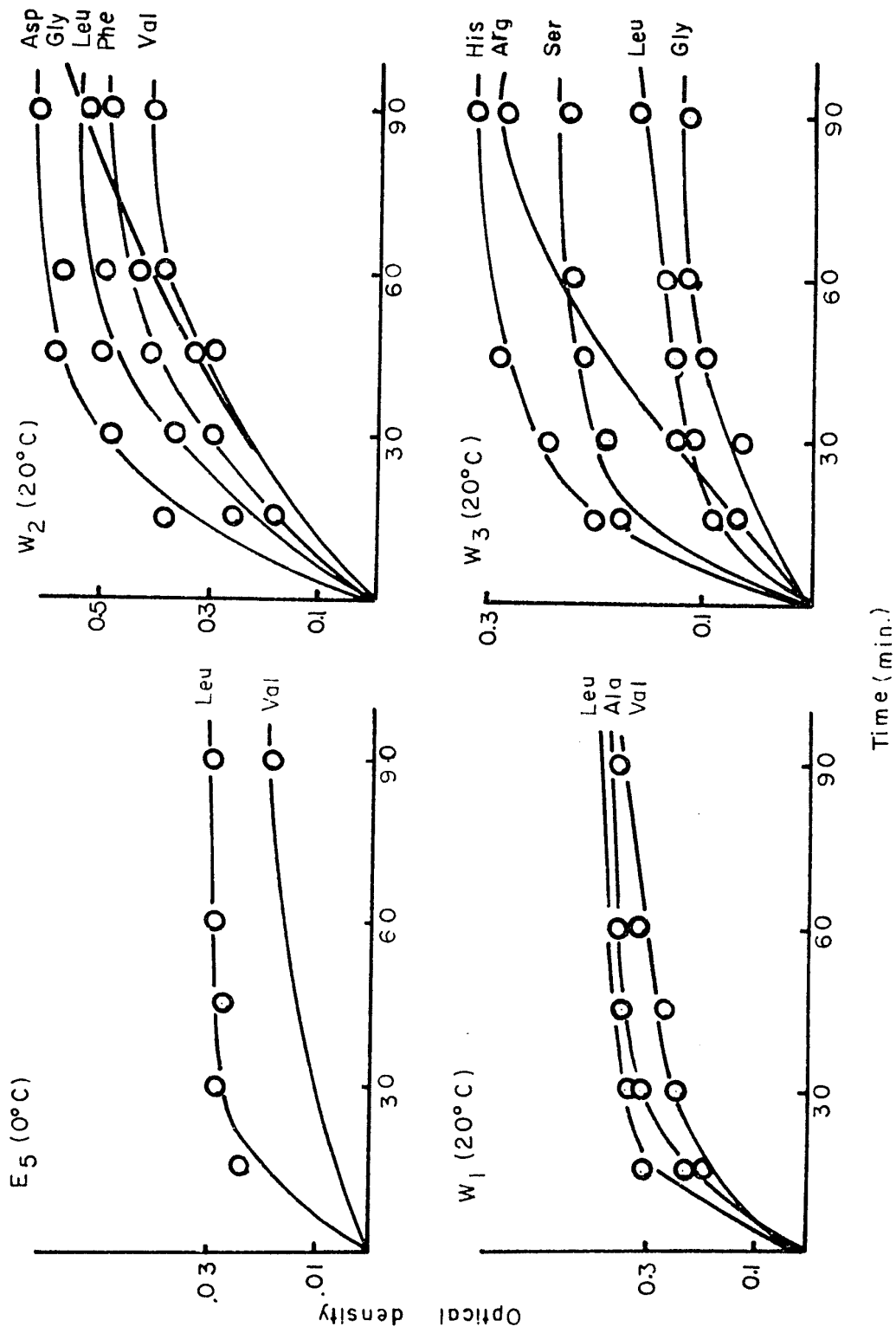
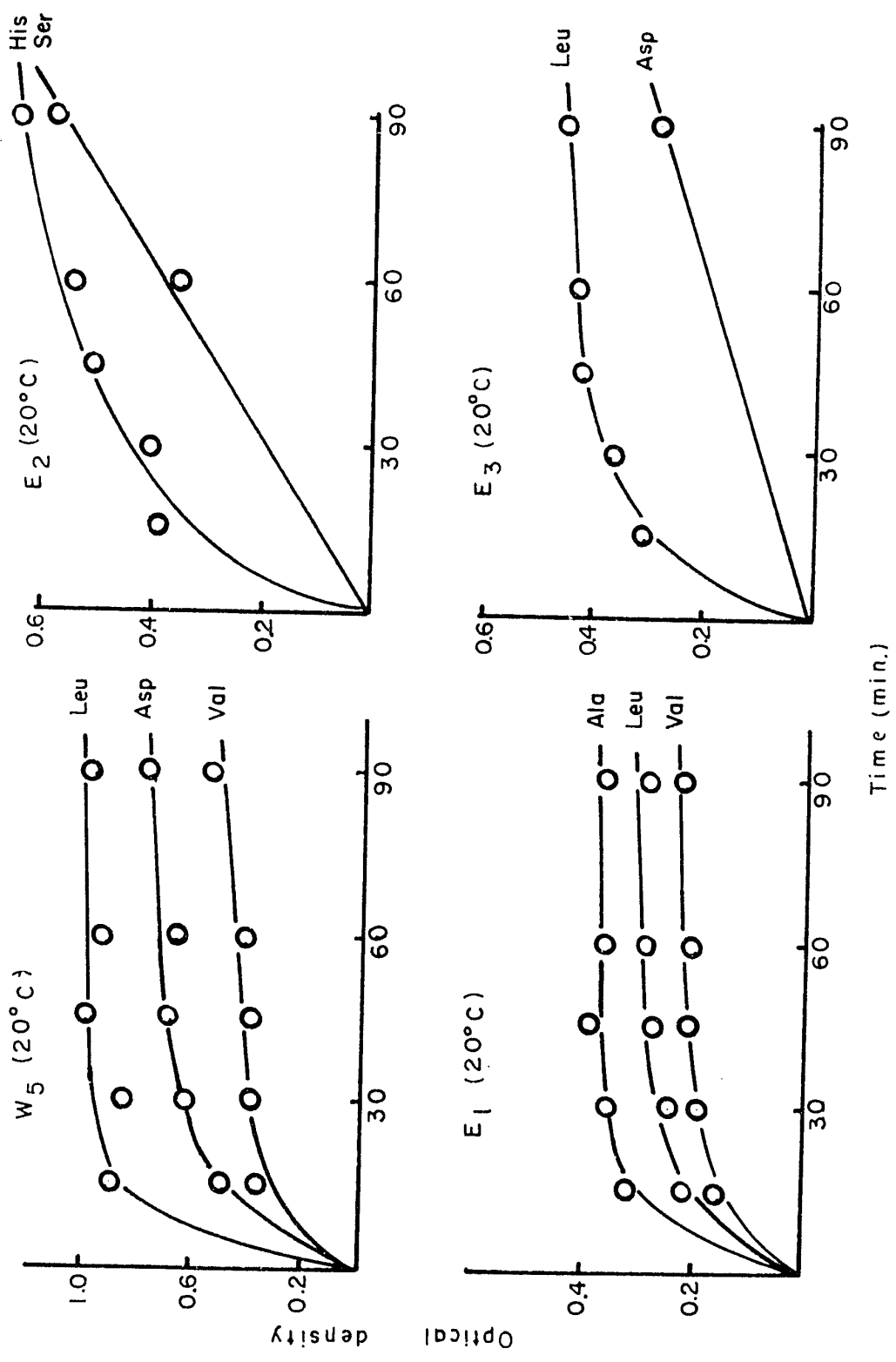


Figure 10. Action of carboxypeptidase on fractions W<sub>5</sub>, E<sub>1</sub>, E<sub>2</sub>, and E<sub>3</sub> from pH 2.0 activation mixture at 20°C



leucyl-valyl (or -leucyl) bond. The amino acid residue preceding leucine could be valine in case of  $W_4$  and aspartic acid in the case of  $W_5$ .

(7) Amino acid analysis of the DNP-derivatives isolated from the activation mixtures

Preliminary work was done on the amino acid content of the DNP-peptides separated by paper chromatography. The method used was only semi-quantitative, therefore the results presented in Table 4 cannot be considered as definitive. Nevertheless they give a good idea about the identity of the amino acids present in each peptide and also about the proportion of each amino acid in a given peptide. The optical density readings were transformed into micromole units using the extinction coefficient (32). The smallest value found for each peptide was arbitrary chosen as 1, therefore the number following each amino acid in Table 4 indicates only the proportion in which each amino acid is present in a given peptide. It is impossible to compare one peptide to another on a quantitative basis, because different quantities of each peptide were used in the analysis. It is evident that a more accurate method of analysis should be used in order to establish the quantitative amino acid composition of the peptides. Nevertheless the results agree well with the hypothesis proposed before that  $W_5$  would be the smallest peptide liberated by cleavage of a peptide bond near the N-terminal end of pepsinogen, and  $W_2$  the biggest one. For example  $W_5$  does not contain any histidine while all the other peptides contain histidine. Recently

Lokshina and Orekhovich (77) have established the N-terminal sequence of pepsinogen, involving 18 amino acid residues: leu-val-leu-glu-pro-ala-glu-phe-ser-leu-lys-asp-gly-lys-val-(asp, pro)-leu-. This amino acid composition agrees very well with that of W<sub>5</sub> and seems to indicate that W<sub>5</sub> comes from the N-terminal end of pepsinogen. It is also important to notice that the peptides W<sub>2</sub> and W<sub>3</sub> contain a high proportion of lysine. It is well known that during conversion of pepsinogen to pepsin there is a disproportionate loss of basic amino acids. Also it should be remembered that the amino acid composition of the inhibitor reveals a high lysine content. It is probable that either W<sub>2</sub> or W<sub>3</sub>, or both can act as inhibitor. Herriott has proposed recently that the lysine residues could be involved in the interaction between the inhibitor and pepsin (78).

The amino acid composition of the ethyl acetate soluble components shows some qualitative and quantitative differences with the amino acid composition of the corresponding water soluble components. This could be due to the fact that these components were not as pure as the water soluble components.

There is one point however which does not agree with the hypothesis as proposed previously. It is the absence of proline in W<sub>4</sub>. If W<sub>4</sub> comes from the N-terminal end of pepsinogen, and is a little longer than W<sub>5</sub>, it should contain proline. The absence of proline could indicate that W<sub>4</sub> does not come from the N-terminal end of pepsinogen, but could be a peptide fragment coming from a section of the pepsinogen chain situated between W<sub>5</sub> and the N-terminal end of pepsin. It could be

liberated by the cleavage of two peptide bonds.

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSION

In the work presented in this thesis, the activation of pepsinogen into pepsin has been studied under different conditions of pH and temperature, in order to elucidate the chemical transformations leading to the production of an active enzyme from an inactive protein. Pepsinogen was purified by a rapid and simple method involving filtration on Sephadex-gel columns. The activation of the purified zymogen was shown to be much faster at pH 2.0 and room temperature than at pH 2.0 and 0°C or at pH 5.6, 20°C or 0°C (Fig. 2 and 2-A). It was also shown that at pH 2.0 the reaction is catalyzed mainly by hydrogen ions while at pH 5.6 the reaction is mainly autocatalytic (Fig. 2-B). In fact only the pH 5.6 and 0°C activation process gave a good straight line, when  $\log \frac{A_0 - A}{A}$  was plotted against time, indicating an autocatalytic reaction. The peptides formed during activation were purified and isolated as their DNP-derivatives by paper chromatography. The main peptide produced at pH 2.0 and at pH 5.6 ( $W_2$ ) was shown to be produced at the same rate as active pepsin, the only exception being activation at pH 5.6 and 0°C where a different peptide  $W_5$  was produced at a higher rate than  $W_2$  (Figs. 3, 4, 5, and 6). The other important peptides produced at pH 2.0 and at pH 5.6,  $W_3$ ,  $W_4$  and  $W_5$ , were present in the activation mixture only in small quantities, their production was fast in the first few minutes of activation and then became very slow or stopped completely; this showed that their production cannot be related to the production of most of the active pepsin present in the activation mixture at pH 2.0 and at pH 5.6. These peptides,  $W_3$ ,  $W_4$ , and  $W_5$ , can not come

from the breakdown of pepsin by autolysis or from the breakdown of  $W_2$ , because their concentration would increase with time as the concentration of  $W_2$  and pepsin increases.

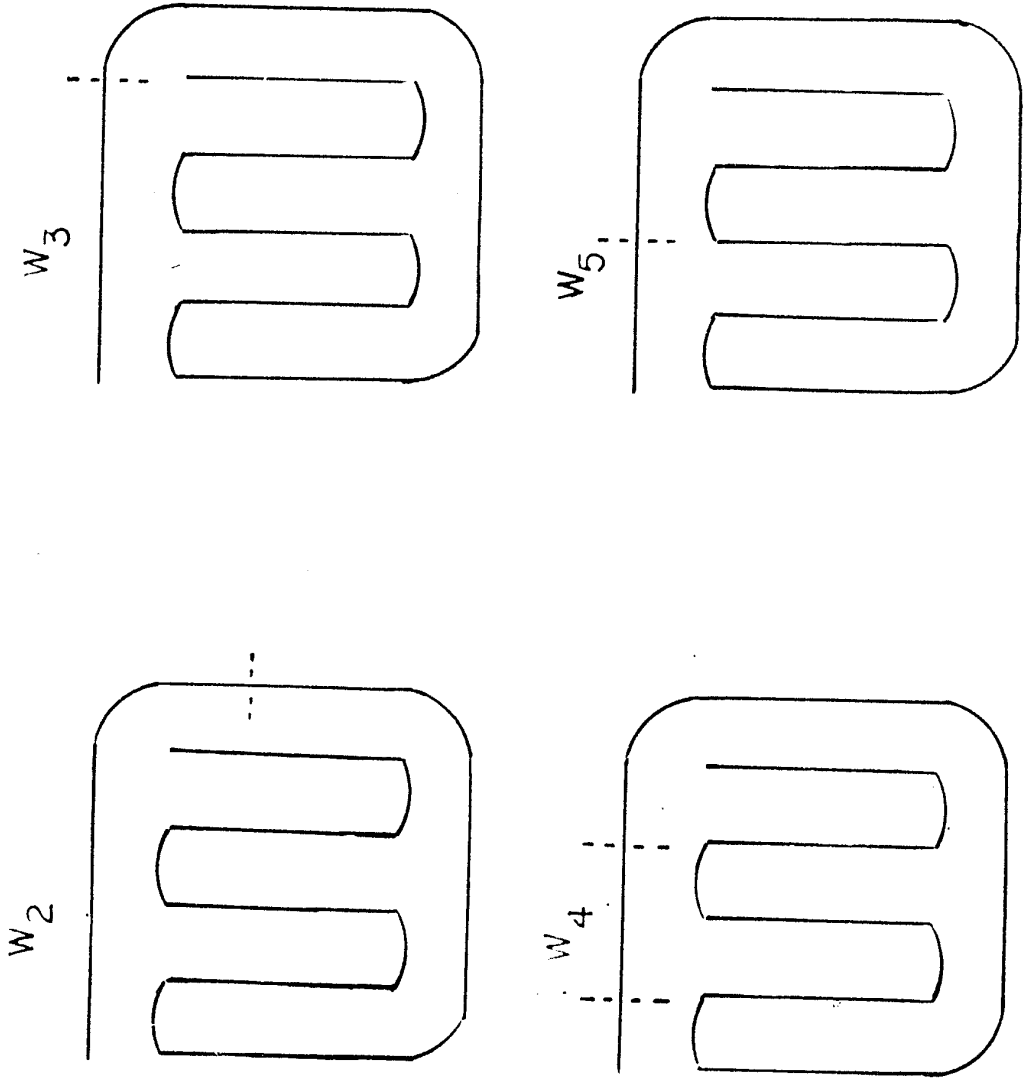
From the study of the C-terminal sequences of pepsinogen and pepsin, it is known that the peptides liberated during activation come from the N-terminal end of pepsinogen. It is possible to imagine that a certain number of peptide bonds in this section of the pepsinogen chain are labile to acid hydrolysis or to peptic hydrolysis, therefore according to the pH of the solution and to the amount of active pepsin present, those different peptide bonds could be cleaved at different rates. The liberation of these peptides would unmask the active center of pepsin and produce the active enzyme. A certain number of different active species of pepsin would be possible according to the peptide bond cleaved in a given molecule; the heterogeneity of pepsin has already been discussed in the introduction. From the amount of  $W_2$  produced at pH 2.0, it seems evident that this peptide is mainly liberated by acid hydrolysis and that one main pepsin species only would be produced at this pH. From N-terminal, C-terminal, and amino acid composition studies, it would seem that the liberation of  $W_2$  would involve cleavage of an aspartyl-isoleucyl peptide bond.

From amino acid composition studies, there is good indication that  $W_5$  would come from the first 18 amino acids of the pepsinogen chain. It is questionable whether the liberation of  $W_5$  produces active pepsin. At pH 5.6 and 0°C,  $W_5$  production is still going up after 30 minutes

(Fig. 6) while the active pepsin production has already stopped (Fig. 2-A). It is possible that the liberation of this short peptide does not unmask completely the active center of pepsin.

In conclusion, we believe that the activation of pepsinogen into active pepsin can be brought about by the cleavage of a certain number of different peptide bonds giving rise to different species of active pepsin. But in each case the cleavage of only one peptide bond would be sufficient to produce the active enzyme. The main peptide bond, cleaves under different conditions, would be an aspartyl-isoleucyl peptide bond leading to the production of one peptide  $W_2$  which could act as the pepsin inhibitor, and to the production of one main type of active pepsin.

Figure 11 Diagrammatic sketch of pepsinogen transformation into pepsin



REFERENCES

1. M. Ravery, C. Fabre, and P. Desmuelle. *Biochim. et Biophys. Acta* 9, 702 (1952).
2. P. Desmuelle and C. Fabre. *Biochim. et Biophys. Acta* 18, 49 (1955).
3. C. Gabeloteau and P. Desmuelle. *Bull. Soc. Chim. Biol.* 40, 35 (1958).
4. J. F. Pechere and H. Neurath. *J. Biol. Chem.* 229, 389 (1957).
5. E. W. Davie and H. Neurath. *J. Am. Chem. Soc.* 74, 6305 (1952).
6. B. Keil, F. Sorm, V. Holeysovky, V. Kostka, B. Meloun, O. Mikes, V. Tomasek, and J. Vanacek. *Collection Czechoslov. Chem. Commun.* 24, 3491 (1959).
7. M. Kunitz. *Enzymologia* 7, 1.
8. J. H. Northrop, M. Kunitz, and R. Herriott. "Crystalline Enzymes" 2nd ed., Columbia Univ. Press, New York, 1948.
9. M. Kunitz. *J. Gen. Physiol.* 22, 293, 429 (1939).
10. F. Tietze. *J. Biol. Chem.* 204, 1 (1953).
11. L. W. Cunningham Jr. *J. Biol. Chem.* 211, 13 (1954).
12. E. F. Jansen and A. K. Balls. *J. Biol. Chem.* 194, 721 (1952).
13. M. Ravery, C. Fabre, and P. Desmuelle. *Biochim. et Biophys. Acta* 12, 547 (1953).
14. E. W. Davie and H. Neurath. *Biochim. et Biophys. Acta* 11, 442 (1953).
15. E. W. Davie and H. Neurath. *J. Biol. Chem.* 212, 515 (1955).
16. C. K. Keith, A. Kazenko, and M. Laskowski. *J. Biol. Chem.* 170, 227 (1947).
17. F. R. Bettelheim. *J. Biol. Chem.* 212, 235 (1955).
18. J. F. Pechere, G. H. Dixon, R. M. Maybury, and H. Neurath. *Biochim. et Biophys. Acta* 31, 259 (1959).
19. B. Moedom. *Biochim. et Biophys. Acta* 31, 260 (1959).

20. M. Rovery, C. Gabeloteau, P. de Vernejoul, A. Guidoni, and P. Desnuelle. *Biochim. et Biophys. Acta* 32, 256 (1958).
21. C. F. Jacobsen. *Compt. rend. trav. lab. Carlsberg Ser. Chim.* 25, 325 (1947).
22. M. Kunitz and J. H. Northrop. *J. Gen. Physiol.* 18, 433 (1935).
23. M. Kunitz. *J. Gen. Physiol.* 22, 207 (1938).
24. F. R. Bettelheim and H. Neurath. *J. Biol. Chem.* 212, 241 (1955).
25. M. Rovery, M. Poilroux, A. Yoshida, and P. Desnuelle. *Biochim. et Biophys. Acta* 23, 608 (1957).
26. J. A. Gladner and H. Neurath. *J. Biol. Chem.* 205, 345 (1953).
27. J. A. Gladner and H. Neurath. *J. Biol. Chem.* 206, 911 (1954).
28. R. M. Herriott. *J. Gen. Physiol.* 21, 501 (1938).
29. R. M. Herriott, V. Desreux, and J. H. Northrop. *J. Gen. Physiol.* 24, 213 (1940).
30. J. H. Northrop. *J. Gen. Physiol.* 13, 739 (1930).
31. J. St. L. Philpot. *Biochem. J.* 29, 2458 (1935).
32. J. H. Northrop. *J. Gen. Physiol.* 30, 177 (1946).
33. J. Steinhardt. *J. Biol. Chem.* 129, 135 (1939).
34. K. Heirnegh and P. Edman. *Biochim. et Biophys. Acta* 24, 219 (1957).
35. M. A. Mitz and R. J. Schlenter. *J. Am. Chem. Soc.* 81, 4024 (1959).
36. E. A. Peterson and H. A. Sober. *J. Am. Chem. Soc.* 78, 751 (1956).
37. V. N. Orechovich et al. *Intern. Congr. Biochem. 5th Congr. Moscow 1961 Symposium No. 4.*
38. V. M. Stepanov et al. *Compt. rend. acad. sci. URSS* 136, 1238 (1961).
39. R. Arnon and G. E. Perlmann. *J. Biol. Chem.* 238, 653 (1963).
40. A. Ryle. *Biochem. J.* 75, 145 (1960).
41. H. Z. Holter. *Physiol. Chem.* 196, 1 (1931).

42. J. H. Northrop. *J. Gen. Physiol.* 15, 29 (1931).
43. R. M. Herriott, V. Desreux, and J. H. Northrop. *J. Gen. Physiol.* 23, 439 (1940).
44. H. Neumann and N. Sharon. *Biochim. et Biophys. Acta* 41, 37 (1960).
45. K. Tokuyasu and M. Funatsu. *J. Biochem. (Tokyo)* 49, 297 (1961).
46. J. H. Northrop. *J. Gen. Physiol.* 5, 263 (1922).
47. J. S. Fruton and M. Bergmann. *Science* 87, 557 (1938).
48. C. R. Harington and R. V. Pitt-Rivers. *Biochem. J.* 38, 417 (1944).
49. F. Sanger and H. Tuppy. *Biochem. J.* 49, 481 (1951).
50. F. Sanger and E. O. P. Thompson. *Biochem. J.* 53, 366 (1953).
51. J. L. Bailey, S. Moore, and W. H. Stein. *J. Biol. Chem.* 221, 143 (1956).
52. C. H. Li. *Advanced Protein Chem.* 11, 101 (1956).
53. V. N. Orekhovich and V. O. Shpikiter. *Doklady Akad. Nauk. SSSR.* 111, 401 (1956).
54. H. Tanber and I. S. Kleiner. *Z. Physiol. Chem.* 220, 205 (1933).
55. D. S. Yasnoff and H. B. Bull. *J. Biol. Chem.* 200, 619 (1953).
56. H. A. Dieu and H. B. Bull. *J. Am. Chem. Soc.* 71, 450 (1949).
57. J. D. Bernal and D. Crowfoot. *Nature* 133, 794 (1934).
58. G. E. Perlmann. *Advances in Protein Chem.* 10, 23 (1955).
59. R. Ege. *Z. Physiol. Chem.* 224, 129 (1934).
60. J. H. Northrop. *J. Gen. Physiol.* 16, 33 (1932).
61. P. D. Royer, H. Lardy, and K. Myrback. "The Enzymes" vol. 4, page 90, 2nd Ed., Academic Press, New York, 1960.
62. H. Van Vunakis and R. M. Herriott. *Biochim. et Biophys. Acta* 23, 600 (1957).

63. O. O. Blumenfeld and G. E. Perlmann. *J. Gen. Physiol.* 42, 553 (1959).
64. F. Sanger. *Biochem. J.* 39, 507 (1945).
65. M. B. Williamson and J. M. Passman. *J. Biol. Chem.* 199, 121 (1952); 222, 151 (1956).
66. P. Edman. *Acta Chem. Scand.* 4, 233 (1950).
67. R. M. Herriott. In "The Mechanism of Enzyme Action", (W.D. McElroy and B. Glass, eds.), Johns Hopkins Press, Baltimore, Maryland, 1954.
68. H. Van Vunakis and R. M. Herriott. quoted in C. B. Anfinsen and R. R. Redfield. *Advanced in Protein Chem.* 11, 1 (1956).
69. M. B. Williamson and J. M. Passman. *Biochim. et Biophys. Acta* 15, 746 (1954).
70. H. L. Kern. Ph.D. Thesis, John Hopkins Univ. 1953.
71. R. M. Herriott. *J. Gen. Physiol.* 22, 65 (1938).
72. R. M. Herriott, Q. R. Bartz, and J. H. Northrop. *J. Gen. Physiol.* 21, 575 (1938).
73. R. M. Herriott. *J. Gen. Physiol.* 24, 325 (1941).
74. H. Van Vunakis and R. M. Herriott. *Biochim. et Biophys. Acta* 22, 537 (1956).
75. O. V. Lobachevskaya. *Ukrain Biokhim. Zhur.* 28, 385 (1956).
76. L. A. Lokshina and V. N. Orekhovich. *J. Biochem. USSR*, English translation, July-August, 655 (1957).
77. L. A. Lokshina and V. N. Orekhovich. *Compt. rend. acad. sci. USSR* 133, 472 (1960).
78. R. M. Herriott. *J. Gen. Physiol.* 45, 57 (1962).
79. S. Blackburn and A. G. Lowther. *Biochem. J.* 48, 126 (1951).
80. G. L. Mills. *Nature* 165, 403 (1950).
81. I. E. Liener. *Biochim. et Biophys. Acta* 37, 522 (1960).
82. J. P. Greenstein and M. Winitz. "Chemistry of the amino acids" Vol. 2, Page 1308, John Wiley and Sons Inc., New York, 1961.